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(54) Title: COMPOSITIONS CONTAINING BETA 2-GLYCOPROTEIN I FOR THE PREVENTION AND/OR TREATMENT OF VASCULAR DISEASE

(57) Abstract: Methods and compositions employing beta2-glycoprotein-1 (2GPI) or derivatives of 2GPI effective in inducing mucosal tolerance to atheroma related antigens, thus inhibiting inflammatory processes contributing to atheromatous vascular disease and sequalae, are provided. Also provided are articles of manufacture comprising mucosal tolerance inducing amounts of beta2-glycoprotein-1 (2GPI) or derivatives thereof.

COMPOSITIONS CONTAINING BETA 2-GLYCOPROTEIN I FOR THE PREVENTION AND/OR TREATMENT OF VASCULAR DISEASE

FIELD OF INVENTION

The present invention relates to an immune-tolerance-inducing composition containing beta-2 glycoprotein I for the prevention and/or treatment of atherosclerosis, and uses thereof.

BACKGROUND OF THE INVENTION

The present invention relates to β2-Glycoprotein I and associated molecules for prevention and treatment of atherosclerosis and related disease and, more particularly, to methods and compositions employing β2-Glycoprotein I and associated molecules effective in inducing immune tolerance and inhibiting inflammatory processes contributing to atheromatous vascular disease and sequalae.

Atherosclerosis

Cardiovascular disease is a major health risk throughout the industrialized world. Atherosclerosis, the most prevalent of cardiovascular diseases, is the principal cause of heart attack, stroke, and gangrene of the extremities, and as such, the principal cause of death in the United States. Atherosclerosis is a complex disease involving many cell types and molecular factors (for detailed reviews, see Ross, 1993, Nature 362: 801-809, Ross, Atherosclerosis 1997, 131Suppl.:S3-7; Schachter, Int J Card 1997;62, Suppl.2:S3-7; Libby, Nature 2002;420:868-74; Zhou et al Exp Opin Biol Ther 2004;4:599-612; Greaves at el, Trends in Immunol. 2002;23:535-41, Martinez-Gonzales et al, Rev Esp Cardiol, 2001;54:218-31, and Faxon et al, Circulation 2004; 109:2617-25). Currently, it is thought that atherosclerosis is the result of a response of the vascular tissues to insult or injury, endothelial dysfunction, and/or inflammation, acting to induce a cellular imbalance, causing normally anticoagulant endothelium with anticoagulant properties becomes prothrombotic (Altman, Thrombosis J. 2003;1:4). The process, which occurs in response to insults to the endothelium and smooth muscle cells (SMCs) of the wall of the artery, consists of the formation of fatty streaks as well as fibrofatty and fibrous lesions or plaques, preceded by and associated with inflammation. The advanced lesions of

atherosclerosis may occlude the artery concerned, and result from an excessive inflammatory-fibroproliferative response to numerous different forms of insult. For example, shear stresses are thought to predispose to the frequent occurrence of atherosclerotic plaques in regions of the circulatory system where turbulent blood flow occurs, such as branch points and irregular structures.

The first observable event in the formation of an atherosclerotic plaque occurs when inflammatory cells such as monocyte-derived macrophages adhere to the vascular endothelial layer and transmigrate through to the sub-endothelial space. Elevated plasma LDL levels lead to lipid engorgement of the vessel walls, with adjacent endothelial cells producing oxidized low density lipoprotein (LDL). In addition, lipoprotein entrapment by the extracellular matrix leads to progressive oxidation of LDL by lipoxygenases, reactive oxygen species, peroxynitrite and/or myeloperoxidase as well as other oxidizing compounds. These oxidized forms of LDLs are then taken up in large amounts by vascular cells through scavenger receptors expressed on their surfaces.

Lipid-filled monocytes and smooth-muscle derived cells are called foam cells, and are the major constituent of the fatty streak. Interactions between foam cells and the endothelial and smooth muscle cells surrounding them produce a state of chronic local inflammation which can eventually lead to activation of endothelial cells, increased macrophage apoptosis, smooth muscle cell proliferation and migration, and the formation of a fibrous plaque (Hajjar, DP and Haberland, ME, J.Biol Chem 1997 Sep 12; 272(37):22975-78). Such plaques occlude the blood vessels concerned and thus restrict the flow of blood, resulting in ischemia, a condition characterized by a lack of oxygen supply in tissues of organs due to inadequate perfusion. When the involved arteries block the blood flow to the heart, a person is afflicted with an acute coronary syndrome [acute myocardial infarction (MI) or unstable angina]; when the brain arteries occlude, the person experiences a stroke. When arteries to the limbs narrow, the result is severe pain, decreased physical mobility, eventually gangrene and possibly the need for amputation.

Involvement of the immune network in atherosclerosis

The recognition that immune mediated processes prevail within atherosclerotic lesions stemmed from the consistent observation of lymphocytes and

macrophages in the earliest stages, namely the fatty streaks. These lymphocytes, which include a predominant population of CD4+ cells (the remainder being CD8+cells), were found to be more abundant over macrophages in early lesions, as compared with the more advanced lesions, in which this ratio tends to reverse. These findings posed questions as to whether the lymphocytes reflect a primary immune sensitization to a possible antigen or alternatively stand as a mere epiphenomenon of a previously induced local tissue damage. Regardless of the factors responsible for the recruitment of these inflammatory cells to the early plaque they seem to exhibit an activated state manifested by concomitant expression of MHC class II HLA-DR and interleukin (IL) receptor as well as leukocyte common antigen (CD45R0) and the very late antigen 1 (VLA-1) integrin.

The on-going inflammatory reaction in the early stages of the atherosclerotic lesion may either be the primary initiating event leading to the production of various cytokines by the local cells (i.e endothelial cells, macrophages, smooth muscle cells and inflammatory cells), or it may be that this reaction is a form of the body's defense immune system towards the hazardous process.

As result of chronic inflammation in atherosclerosis, numerous markers such as CPR (C-reactive protein), cytokines (interleukin-6 and 18, tumor necrosis factor α), adhesion molecules (ICAM-1), E-selectin and acute-phase reactants related to the clotting system (e.g. fibrinogen) are increased in plasma, possible predictors of further cardiovascular events. Interleukin-18 plays a key role in the inflammation cascade and is an important regulator of both innate and acquired immunities. It induces the production of interferon-γ and T-lymphocytes, has been found in human atherosclerotic lesions, and was identified as a strong independent predictor of death from cardiovascular causes in patients with stable as well as unstable angina. Inhibition of interleukin-18 reduced lesion progression with a decrease of inflammatory cells.

Matrix metalloproteinase (MMP-9) (gelatinase B), secreted by macrophages and other inflammatory cells, has been identified in various pathological processes such as general inflammation, tumor metastasis, respiratory diseases, myocardial injury, vascular aneurysms, and remodeling. MMP-9 is elevated in patients with unstable angina. A strong association has been noted between baseline MMP-9

levels and future risk of CV death, independent of IL-18. Combined determination of plasma MMP-9 and IL-18 identifies patients at very high risk.

Proinflammatory cytokines derived from monocytes, macrophages and/or adipose tissue trigger CRP in the liver. C-Reactive protein is an acute-phase reactant, a marker of inflammation, and predicts early and late mortality in patients with acute coronary syndromes. It is an independent predictor of future cardiovascular events. CRP itself promotes inflammation and atherogenesis via effects on monocytes and endothelial cells and increasing the concentration and activity of plasminogen activator inhibitor-1. CRP in atheroma participates in the pathogenesis of unstable angina and restenosis after coronary intervention. Thus, there is a vicious circle: inflammation releases proinflammatory cytokines, which in turn maintain inflammation (Altman Thrombosis J. 2003;1:4).

The cytokines which have been shown to be upregulated by the resident cells include TNF-α, IL-1, IL-2, IL-6, IL-8, IFN-γ, IL-18 and monocyte chemoattractant peptide-1 (MCP-1). Platelet derived growth factor (PDGF) which is expressed by all cellular constituents within atherosclerotic plaques have also been shown to be overexpressed, thus possibly intensifying the preexisting inflammatory reaction by a co-stimulatory support in the form of a mitogenic and chemotactic factor. Recently, Uyemura K et al (J Clin Invest 1996 97; 2130-2138) have elucidated type 1 T-cell cytokine pattern in human atherosclerotic lesions exemplified by a strong expression of IFN-γ but not IL-4 mRNA in comparison with normal arteries. Furthermore, IL-12 - a T-cell growth factor produced primarily by activated monocytes and a selective inducer of Th1 cytokine pattern, was found to be overexpressed within lesions as manifested by the abundance of its major heterodimer form p70 and p40 (its dominant inducible protein) mRNA.

Similar to the strong evidence for the dominance of the cellular immune system within the atherosclerotic plaque, there is also ample data supporting the involvement of the local humoral immune system. Deposition of immunoglobulins and complement components have been shown in the plaques in addition to the enhanced expression of the C3b and C3Bi receptors in resident macrophages. In a recent study, Caligiuri et al disclosed that B cells from apoE ° mice inhibit atherosclerosis in splenectomized and intact mice (Caligiuri et al, J Clin Invest, 2002,

109:745-53). Similarly, studies involving immunization of animals with plaque related antigens indicate the contribution of humoral immunity to attenuation of plaque formation and inhibition of atherosclerosis (see, for example, George et al, Atherosclerosis, 1998, 138;147-152; Zhou et al, Arterioscler Thromb Vasc Biol 2001;21:108-14; and Freigang, et al. 1998;1972-82).

Atherosclerosis and inflammation

Valuable clues with regard to the contribution of immune mediated inflammation to the progression of atherosclerosis comes from animal models. Hence, it seems that immunocompromised mice (class I MHC deficient) tend to develop accelerated atherosclerosis as compared with immune competent mice. Additionally, treatment of C57BL/6 mice (Emeson EE, Shen ML. Accelerated atherosclerosis in hyperlipidemic C57BL/6 mice treated with cyclosporine A. Am J Pathol 1993; 142: 1906-1915) and New-Zealand White rabbits (Roselaar SE, Schonfeld G, Daugherty A. Enhanced development of atherosclerosis in cholesterol fed rabbits by suppression of cell-mediated immunity. J Clin Invest 1995; 96: 1389-1394) with cyclosporine A, which is a potent suppressor of IL-2 transcription resulted in a significantly enhanced atherosclerosis under "normal" lipoprotein "burden". More recently, it has been demonstrated that cyclosporin A-related autoreactive mechanisms contribute to the high incidence of graft vasculopathy (Chen, Cli Immunol 2001;100:57-70). These latter studies may provide insight into the possible roles of the immune system as engaged in counteracting the self-perpetuating inflammatory process within the atherosclerotic plaque.

Oxidized LDL has been implicated in the pathogenesis of atherosclerosis and atherothrombosis, by it's action on monocytes and smooth muscle cells, and by inducing endothelial cell apoptosis, impairing anticoagulant balance in the endothelium. Oxidized LDL also inhibits anti-atherogenic HDL-associated breakdown of oxidized phospholipids (Mertens, A and Holvoet, P, FASEB J 2001 Oct; 15(12):2073-84). This association is also supported by many studies demonstrating the presence of oxidized LDL in the plaques in various animal models of atherogenesis; the retardation of atherogenesis through inhibition of oxidation by pharmacological and/or genetic manipulations; and the promising results of some of the interventional trials with anti-oxidant vitamins (see, for example, Witztum J and

Steinberg, D, Trends Cardiovasc Med 2001 Apr-May;11(3-4):93-102 for a review of current literature). Indeed, oxidized LDL and malondialdehyde (MDA)-modified LDL have been recently proposed as accurate blood markers for 1st and 2nd stages of coronary artery disease (US Pat. Nos. 6,309,888 to Holvoet et al and 6,255,070 to Witztum, et al).

Reduction of LDL oxidation and activity has been the target of a number of suggested clinical applications for treatment and prevention of cardiovascular disease. Bucala, et al (US Pat. No. 5869534) discloses methods for the modulation of lipid peroxidation by reducing advanced glycosylation end product, lipid characteristic of age-, disease- and diabetes-related foam cell formation. Tang et al, at Incyte Pharmaceuticals, Inc. (US Pat. No. 5,945,308) have disclosed the identification and proposed clinical application of a Human Oxidized LDL Receptor in the treatment of cardiovascular and autoimmune diseases and cancer.

Beta $_2$-Glycoprotein I

Another abundant atherogenesis-related plaque component is Beta $_2$-Glycoprotein I. Beta $_2$-Glycoprotein I (β_2GPI) is a 50-kDa molecule that acts as an anticoagulant in *in-vitro* assays. Although the exact role of β_2GPI in atherogenesis has yet to be elucidated, several relevant properties have been observed: 1) it is able to bind immobilized negatively charged phospholipids or phospholipid-expressing cells (apoptotic cells, activated platelets); 2) it is able to bind to modified cellular surfaces, enhancing their clearance by scavenging macrophages (Chonn A, et al J Biol Chem 1995; 270: 25845-49; and Thiagarajan P, et al Arterioscler Thromb Vasc Biol 1999; 19:2807-11); and 3) it is an important target for binding of autoimmune antiphospholipid antibodies (aPLs). β_2GPI has to undergo structural alteration in order to be recognized by aPLs. This alteration may be initiated, for example, by binding to negatively charged phospholipids or high-binding plates, but also *in-vivo* by binding apoptotic cells that express phophatidylserine.

Recent studies investigating the importance of anti β_2GPI antibodies in promoting a procoagulant state have focused on the effects of these antibodies on cellular and protein components of the coagulation system (endothelial cells, platelets and macrophages; tissue factor and coagulation factors). These studies indicate that anti β_2GPI antibodies prevent the deactivation of platelets, sustaining their

phagocytic clearance; interact with late endosomes of human endothelial cells; and suppress the inhibitory activity of the tissue factor pathway inhibitor. This association with coagulation events is consistent with β_2GPIs proposed function in the prothrombotic antiphospholipid syndrome (APLS). US Pat Nos 5998223 and 5344758 (to Matsuura, et al and Krilis, et al, respectively), and US Patent Application Nos. 20030100036 and 10/488688 to Vojdani et al. and Matsuura et al., respectively, disclose the application of anti β_2GPI antibodies, some to cryptic epitopes, for diagnostics in APLS and SLE. US Patent No. 5,900,359 to Matsuura et al teaches the use of anti-β_2GPI for the detection of circulating oxidized LDL via Ox-LDL-β_2GPI complexes. U.S. Patent Application No. 10/694033 to Berg et al. discloses the detection of anti-β_2GPI antibodies for early diagnosis of activation of coagulation response in vascular and clotting disorders. Koike et al (U.S. Patent Application No. 10/429,479) teaches the determination of nicked β_2GPI in blood samples for diagnosing cerebral infarct. However, no therapeutic applications are disclosed by the authors.

The antigenic properties of β_2GPI-cardiolipin complex, and their association with anti-PL antibody related diseases has led some researchers to propose the use of β_2GPI or β_2GPI sequences as B-cell toleragens in the treatment of anti-PL antibody related disease such as recurrent stroke and recurrent fetal loss (see U.S. Patent Application No. 10/044844 and U.S. Patent No. 5,874,409 to Victoria et al.).

Since aminophospholipids have been identified as readily accessible markers in the walls of tumor blood vessels, the application of anti-β_2GPI antibodies for therapy in cancer and tumorigenesis has been proposed (see, for example, Thorpe et al U.S. Patent Application Nos. 09/990,833 and 10/259,244, and U.S. Patents No. 6,818,213 and 6,783,760). The use of β_2GPI in an anti-cancer vaccine is taught by Schroit in U.S. Patent No. 6,806,354 and U.S. Patent Application No. 09/974,753.

Immunization of atherosclerosis-prone LDL R-/- mice by subcutaneous injection of human β_2GPI emulsified in complete Freunds adjuvant resulted in high titers of anti-β_2GPI antibodies, detectable amounts of circulating immune complexes with β_2GPI, and induction of increased plaque formation and other indicators of early atherogenesis (George et al, Circulation 1998; 98: 1108-1115).

Heat shock protein (HSP)

A third important plaque-related component associated with atherogenesis is the 60/65 kDa heat shock protein (HSP). This mitochondrial protein is a member of the HSP family, which constitutes nearly 24 proteins displaying high degree of sequence homologies between different species. These proteins, as their name implies, are expressed in response to stresses including exposure to free radicals, heat, mechanical shear stress, infections and cytokines, and protect against unfolding and denaturation of cellular proteins. This has led to their designation as molecular 'chaperones'. However, HSP function may have undesired consequences, since over expression of HSPs may, under certain conditions promote an autoimmune reaction with resultant tissue damage. The mechanisms responsible for the HSP immune mediated damage are as yet unclear: it is presumed that cryptic, "non-self" neo-epitopes are exposed following their upregulation. Alternatively, it was suggested that cross-reaction exists between self-HSP and 'foreign' HSP epitopes introduced following infections which may trigger a pathological, autoimmune response against native HSP. Support for the involvement of HSP in autoimmunity is provided by studies documenting enhanced autoantibody as well as cellular response to HSP 60/65 in several autoimmune diseases (Schoenfeld, Y et al Autoimmunity 2000 Sep; 15(2):199-202; US Pat No. 6130059 to Covacci, et al; and Gromadza G, et al Cerebrovascul Dis 2001, Oct; 12(3):235-39).

The link between HSP 65 and atherosclerosis was initially recognized by George Wick's group, who found that normocholesterolemic rabbits immunized with different antigens developed atherosclerosis, provided the preparation used for immunization contained complete Freund's adjuvant (CFA)(Xu, Q, et al Arterioscler Thromb 1992;12:789-99). Since the major constituent of CFA is heat killed mycobacterium tuberculosis, the principal component of which is the HSP-65, they reasoned that the immune response towards this component led to the development of atherosclerosis. This was confirmed when these authors demonstrated that immunization of animals with HSP 65 produced pronounced atherosclerosis, and that T cells from experimentally atherosclerotic rabbits overexpressed HSP-65, indicating a localized immune reaction restricted to the stressed arterial vessel. The importance of endogenous HSP-65 in atherogenesis was further demonstrated by the acceleration

of fatty streak formation following HSP-65 (or Mycobacterium tuberculosis) immunization of naïve mice (George J, et al Arterioscler Thromb Vasc Biol 1999; 19:505-10;).

Involvement of humoral immune mechanisms in response to HSP-65 were observed in atherosclerosis: a marked correlation has been found between high levels of anti-HSP65 antibodies and the extent of sonographically estimated carotid narrowing in a screen of healthy individuals (Xu Q. et al Lancet 1993; 341: 255-9; Xu Q. et al Circulation 1999; 100(11):1169-74). In addition, in-vitro experiments with cultured endothelial cells have demonstrated the concentration and time dependent induction of endothelial cell adhesion to monocytes and granulocytes following incubation with HSP65.

Atherosclerosis and autoimmune disease

Because of the presumed role of the excessive inflammatory-fibroproliferative response in atherosclerosis and ischemia, a growing number of researchers have attempted to define an autoimmune component of vascular injury. In autoimmune diseases the immune system recognizes and attacks normally non-antigenic body components (autoantigens), in addition to attacking invading foreign antigens. The autoimmune diseases are classified as auto- (or self-) antibody mediated or cell mediated diseases. Typical autoantibody mediated autoimmune diseases are myasthenia gravis and idiopathic thrombocytopenic purpura (ITP), while typical cell mediated diseases are Hashimoto's thyroiditis and type I (Juvenile) Diabetes.

Atherosclerosis is not a classical autoimmune disease, although some of its manifestations such as the production of the plaque that obstructs the vasculature may be related to aberrant immune responsiveness. In classical autoimmune disease, one can often define very clearly the sensitizing autoantigen attacked by the immune system and the component(s) of the immune system which recognize the autoantigen (humoral, i.e. autoantibody or cellular, i.e. lymphocytes). Above all, one can show that by passive transfer of these components of the immune system the disease can be induced in healthy animals, or in the case of humans the disease may be transferred from a sick pregnant mother to her offspring. Many of the above are not prevailing in

atherosclerosis. Atherosclerosis, and its related conditions, can by no means be considered a classic autoimmune disease.

Indeed, much of the prior art teaches away from the inclusion of atherosclerosis as a classic autoimmune disease. Autoimmune diseases or conditions are defined as those in which an immune response (humoral or cellular) possess pathogenic properties that should be either identified in an autoimmune state or be transferable to non-immune animals (Harrison's Textbook of Internal Medicine, Autoimmune Diseases).

Atherosclerosis progresses gradually and does not have the classic flare and remission of classic autoimmune disease. Indeed, unlike other autoimmune diseases, atherosclerosis does not respond to corticosteroids or immune suppressants: treatment with cyclosporin A further aggravates the disease (Emeson et al Am J Pathol 1993;142: 1906-15). In fact, Meir et al, in a recent review of the contribution of inflammation to atherosclerosis in humans (Commentaries, Int. Atheroscler Soc.) concluded that "thus far there is neither cogent clinical evidence that anti-inflammatory agents decrease vascular morbidity or mortality, nor cogent evidence linking them to decreased atherogenesis in humans. Inflammation may simply be a marker of active disease". In addition, the disease definitely has common risk factors such as hypertension, diabetes, lack of physical activity, smoking and others, the disease affects elderly people and has a different genetic preponderance than in classical autoimmune diseases.

Treatment of inflammatory disease may be directed towards suppression or reversal of general and/or disease-specific immune reactivity. Thus Aiello, for example (US Pat. Nos. 6,034,102 and 6,114,395) discloses the use of estrogen-like compounds for treatment and prevention of atherosclerosis and atherosclerotic lesion progression by inhibition of inflammatory cell recruitment. Similarly, Medford et al (US Pat. No. 5,846,959) disclose methods for the prevention of formation of oxidized PUFA, for treatment of cardiovascular and non-cardiovascular inflammatory diseases mediated by the cellular adhesion molecule VCAM-1. Furthermore, Falb (US Pat. No. 6,156,500) designates a number of cell signaling and adhesion molecules abundant in atherosclerotic plaque and disease as potential targets of anti-inflammatory therapies. Colon-Cruz et al. (US Patent No. 6,821,964) teach the use of chemokines receptor modulators (CCR2 and CCR3 antagonists) for

treatment of atherosclerosis. Tracey (US Patent No. 6,610,713) teaches treatment of atherosclerosis by the inhibition of inflammatory cytokines release with cholinergic agonists and vagus nerve stimulation. Benyunes et al (US Patent Application No. 10/818765) discloses the use of a surface marker-targeted B-cell antagonist for boosting the inhibition of TNF-γ in autoimmune disease.

Since oxidized LDL, β_2GPI and HSP 65 have been clearly implicated in the pathogenesis of atherosclerosis (see above), the contribution of these prominent plaque components to autoimmunity in atheromatous disease processes has been investigated.

Immune responsiveness to plaque associated molecules

It is known that Ox LDL is chemotactic for T-cells and monocytes.

Ox LDL and its byproducts are also known to induce the expression of factors such as monocyte chemotactic factor 1, secretion of colony stimulating factor and platelet activating properties, all of which are potent growth stimulants.

The active involvement of the cellular immune response in atherosclerosis has been substantiated (see, for example, Stemme S, et al, Proc Natl Acad Sci USA 1995; 92: 3893-97), by detection of isolated CD4+ within plaques clones responding to Ox LDL as stimuli. The clones corresponding to Ox LDL (4 out of 27) produced principally interferon-γ rather than IL-4. It remains to be seen whether the above T-cell clones represent mere contact of the cellular immune system with the inciting strong immunogen (Ox LDL) or that this reaction provides means of combating the apparently indolent atherosclerotic process.

The data regarding the involvement of the humoral mechanisms and their meaning are much more controversial. One recent study reported increased levels of antibodies against MDA-LDL, a metabolite of LDL oxidation, in women suffering from heart disease and/or diabetes (Dotevall, et al., Clin Sci 2001 Nov; 101(5): 523-31). Other investigators have demonstrated antibodies recognizing multiple epitopes on the oxidized LDL, representing immune reactivity to the lipid and apolipoprotein components (Steinerova A, et al., Physiol Res 2001;50(2): 131-41) in atherosclerosis and other diseases, such as diabetes, renovascular syndrome, uremia, rheumatic fever and lupus erythematosus. Several reports have associated increased levels of antibodies to Ox LDL with the progression of atherosclerosis (expressed by the

degree of carotid stenosis, severity of peripheral vascular disease etc.). Most recently, Sherer et al (Cardiology 2001;95(1):20-4) demonstrated elevated levels of antibodies to cardiolipin, β-2GPI and oxLDL, but not phosphatidylcholine or endothelial cells in coronary heart disease. Thus, there seems to be a consensus as to the presence of anti-plaque- component antibodies in the form of immune complexes within atherosclerotic plaque, but uncertainty as to their role in atherogenesis.

Regarding the immunogenicity of β_2GPI, it has been shown that β_2GPI serves as a target antigen for an immune-mediated attack, influencing the progression of atherosclerosis in humans and mice. George J et al. immunized LDL-receptor deficient mice with β_2GPI, producing a pronounced humoral immune response to human Beta2GPI, and larger early atherosclerotic lesions in comparison with controls (George J, et al Circulation 1998; 15:1108-15). Afek A, et al obtained similar results in atherosclerosis- prone apolipoprotein-E-knockout mice immunized once with human β_2GPI and fed a high fat diet for 5 weeks (Afek A et al. Pathobiology 1999;67:19-25).

Further, although immune reactivity to β_2GPI in humans with the prothrombotic antiphospholipid syndrome has traditionally been attributed to the presence of autoantibodies to β_2GPI, recent observations have indicated the importance of a cellular immune response to β_2GPI. T-cells reactive with β_2GPI have been demonstrated in the peripheral blood of patients with antiphospholipid syndrome. These T cells displayed a T-helper-1 phenotype (secreting the proinflammatory (and proatherogenic) cytokine interferon-) and were also capable of inducing tissue factor production (Visvanathan S, and McNiel HP. J Immunolog 1999; 162:6919-25). Taken together, the abundant data gathered to date regarding anti β_2GPI (for review see Roubey RA, Curr Opinion Rheumatol 2000; 12:374-378), indicates that the immune response to this plaque related antigen may play a significant role in influencing the size and composition of atherosclerotic plaque.

Finally, there exists a significant dependency between the antigenicity, and pathogenicity of oxidized phospholipids and β_2GPI. As mentioned above, some of the autoimmune epitopes associated with minimally modified LDL and β_2GPI are cryptic. Kyobashi, et al (J Lipid Res 2001; 42:697-709), and Koike, et al (Ann Med 2000; 32:Suppl I 27-31) have identified a macrophage-activating oxLDL specific

ligand present only with β_2GPI- OxLDL complex formation. This ligand was recognized by APLS-specific autoantibodies. Thus, there is evidence from both laboratory and clinical studies for the pathogenic role of β_2GPI and other plaque components, and their importance as autoantigens in atherosclerosis, as well as other diseases.

Mucosal Tolerance in Treatment of Autoimmune Disease

Recently, new methods and pharmaceutical formulations have been found that are useful for treating autoimmune diseases (and related T-cell mediated inflammatory disorders such as allograft rejection and retroviral-associated neurological disease). These treatments induce tolerance, orally or mucosally, e.g. by inhalation, using as tolerizers autoantigens, bystander antigens, or disease-suppressive fragments or analogs of autoantigens or bystander antigens. Such treatments are described, for example, in US Patent Nos. 5,935,577, 6,019,970, 6,790,447, 6,703,361, 6,645,504, 5,961,977, 6,077,509, to Weiner et al., 5,843,449 to Boots et al., and US Patent Applications 10/451,370, 10/989,724 and Israel Patent Application No. 126447 to Harats et al. Autoantigens and bystander antigens are defined below (for a general review of mucosal tolerance see Nagler-Anderson, C., Crit Rev Immunol 2000;20(2):103-20, and Weiner et al. Microb Infect 2001;3:947-54). Intravenous administration of autoantigens (and fragments thereof containing immunodominant epitopic regions of their molecules) has been found to induce immune suppression through a mechanism called clonal anergy. Clonal anergy causes deactivation of only immune attack T-cells specific to a particular antigen, the result being a significant reduction in the immune response to this antigen. Thus, the autoimmune response-promoting T-cells specific to an autoantigen, once anergized, no longer proliferate in response to that antigen. This reduction in proliferation also reduces the immune reactions responsible for autoimmune disease symptoms (such as neural tissue damage that is observed in multiple sclerosis; MS). There is also evidence that oral administration of autoantigens (or immunodominant fragments) in a single dose and in substantially larger amounts than those that trigger "active suppression" may also induce tolerance through anergy (or clonal deletion).

A method of treatment has also been disclosed that proceeds by active suppression. Active suppression functions via a different mechanism from that of

clonal anergy. This method, discussed extensively in PCT Application PCT/US93/01705, involves oral or mucosal administration of antigens specific to the tissue under autoimmune attack. These are called "bystander antigens". This treatment causes regulatory (suppressor) T-cells to be induced in the gut-associated lymphoid tissue (GALT), or bronchial associated lymphoid tissue (BALT), or most generally, mucosa associated lymphoid tissue (MALT) (MALT includes GALT and BALT). These regulatory cells are released in the blood or lymphatic tissue and then migrate to the organ or tissue afflicted by the autoimmune disease and suppress autoimmune attack of the afflicted organ or tissue. The T-cells elicited by the bystander antigen (which recognize at least one antigenic determinant of the bystander antigen used to elicit them) are targeted to the locus of autoimmune attack where they mediate the local release of certain immunomodulatory factors and cytokines, such as transforming growth factor beta (TGF beta), interleukin-4 (IL-4), and/or interleukin-10 (IL-10). Of these, TGF-beta is an antigen-nonspecific immunosuppressive factor in that it suppresses immune attack regardless of the antigen that triggers the attack. (However, because oral or mucosal tolerization with a bystander antigen only causes the release of TGF-beta in the vicinity of autoimmune attack, no systemic immunosuppression ensues.) IL-4 and IL-10 are also antigen-nonspecific immunoregulatory cytokines. IL-4 in particular enhances (T helper 2) Th_2 response, i.e., acts on T-cell precursors and causes them to differentiate preferentially into Th_2 cells at the expense of Th_1 responses. IL-4 also indirectly inhibits Th_1 exacerbation. IL-10 is a direct inhibitor of Th_1 responses. After orally tolerizing mammals afflicted with autoimmune disease conditions with bystander antigens, increased levels of TGF-beta, IL-4 and IL-10 are observed at the locus of autoimmune attack (Chen, Y. et al., Science, 265:1237-1240, 1994). The bystander suppression mechanism has been confirmed by von Herreth et al.(J. Clin. Invest., 96:1324-1331, September 1996).

More recently, oral tolerance has been effectively applied in treatment of animal models of inflammatory bowel disease by feeding probiotic bacteria (Dunne, C, et al., Antonie Van Leeuwenhoek 1999 Jul-Nov;76(1-4):279-92), autoimmune glomerulonephritis by feeding glomerular basement membrane (Reynolds, J. et al., J Am Soc Nephrol 2001 Jan;12(1): 61-70) experimental allergic encephalomyelitis

(EAE, which is the equivalent of multiple sclerosis or MS), by feeding myelin basic protein (MBP), adjuvant arthritis and collagen arthritis, by feeding a subject with collagen and HSP-65, respectively. A Boston based company called Autoimmune has carried out several human experiments for preventing diabetes, multiple sclerosis, rheumatoid arthritis and uveitis. The results of the clinical trials have been less impressive than the animal experiments, however there has been some success with the prevention of arthritis.

Oral tolerance to autoantigens found in atherosclerotic plaque lesions has also been investigated. Study of the epitopes recognized by T-cells and Ig titers in clinical and experimental models of atherosclerosis indicated three candidate antigens for suppression of inflammation in atheromatous lesions: oxidized LDL, the stress-related heat shock protein HSP 65 and the cardiolipin binding protein β_2GPI. US Patent Application 09/806,400 to Shoenfeld et al (filed Sept 30, 1999), and US Patent Application Nos. 10/451,370 and 10/989,724 to Harats et al., which are incorporated by reference herein in there entirety, disclose the reduction by approximately 30% of atherogenesis in the arteries of genetically susceptible LDL receptor deficient mice (LDL-RD) fed oxidized human LDL and other atheroma related antigens. Although significant inhibition of atherogenesis was achieved, presumably via oral tolerance, no identification of specific lipid antigens or immunogenic LDL components was made. Another obstacle encountered was the inherent instability of the orally fed antigen in vivo, due to digestive breakdown, and uptake of oxidized LDL by the liver and cellular immune mechanisms. It is plausible that a mucosal route of administration other than feeding (oral) would have provided tolerance of greater efficiency.

The induction of immune tolerance and subsequent prevention or inhibition of autoimmune inflammatory processes has been demonstrated using exposure to suppressive antigens via mucosal. The membranous tissue around the eyes, the middle ear, the respiratory and other mucosa, and especially the mucosa of the nasal cavity, like the gut, are exposed to many invading as well as self- antigens and possess mechanisms for immune reactivity. Thus, Rossi, et al (Scand J Immunol 1999 Aug;50(2):177-82) found that nasal administration of gliadin was as effective as intravenous administration in downregulating the immune response to the antigen

in a mouse model of celiac disease. Similarly, nasal exposure to acetylcholine receptor antigen was more effective than oral exposure in delaying and reducing muscle weakness and specific lymphocyte proliferation in a mouse model of myasthenia gravis (Shi, FD. et al, J Immunol 1999 May 15; 162 (10): 5757-63), intranasal or aerosol administration of pancreatic islet autoantigen in prediabetic mice reduced the incidence of diabetes (Hanninen et al., Immunol Rev 2000;173:109-19), intranasal administration of Staph enterotoxin A protected mice against toxic shock syndrome (Collins, et al., Infect Immun 2002;70:2282-87, and nasal tolerance to E-selectin inhibited ischemic and hemorrhagic stroke in hypertensive stroke-prone (SHR-SP) rats (Takeda, et al., Stroke 2002;33:2156). Therefore, immunogenic compounds intended for mucosal as well as intravenous or intraperitoneal administration should be adaptable to oral, nasal and other membranous routes of administration.

The current treatments for the prevention and treatment of atherosclerosis include certain pharmacological approaches, in addition to alteration of lifestyle factors which can ameliorate atherosclerosis, such as diet control, weight loss, increased exercise, and smoking cessation. Examples of pharmacological agents in current use for the treatment and prevention of atherosclerosis are hydroxymethylglutaryl-coenzyme A (HMGCoA) reductase inhibitors (statins) to control high LDL, nicotinic acid to control high lipoprotein (a) and low high density lipoprotein (HDL), and fibric acid derivatives to control high levels of triglycerides. Adjunctive pharmacological treatment includes measures directed toward control of diabetes mellitus and hypertension.

In view of the foregoing, a need still exists to develop methods and compositions for treating and/or preventing vascular disorders such as atherosclerosis. Preferably, such methods and compositions would include non-invasive modes of administration and, more preferably, be based, in part, on the molecular interactions which mediate an inflammatory response.

Thus, there is clearly a need for novel methods of employing, and compositions containing β2 glycoprotein I capable of superior tolerizing immunogenicity in mucosal administration.

SUMMARY OF THE INVENTION

The teachings of George et al., Cardiovascular Research 2004, 62:603-609, entitled "Suppression of early atherosclerosis in LDL receptor deficient mice by oral tolerance with beta2 glycoprotein I" are also incorporated by reference as if fully set forth herein.

According to one aspect of the present invention there is provided a method for prevention and/or treatment of a vascular condition in a subject in need thereof, the method effected by administering to a mucosal surface of the subject a mucosal tolerance-inducing amount of an active ingredient selected from the group consisting of beta$_2$-glycoprotein-1 (β_2GPI) or a derivative of β_2GPI, thereby inducing mucosal tolerance.

According to further features in preferred embodiments of the invention described below the vascular condition is selected from the group consisting of atherosclerosis, cardiovascular disease, cerebrovascular disease, peripheral vascular disease, stenosis, restenosis and/or in-stent-stenosis.

According to an additional aspect of the present invention there is provided a method for modulating an immune response to a beta$_2$-glycoprotein-1 (β_2GPI) in a subject in need thereof, the method effected by administering to a mucosal surface of the subject a mucosal tolerance-inducing amount of an active ingredient selected from the group consisting of beta$_2$-glycoprotein-1 (β_2GPI) or a derivative of β_2GPI, thereby inducing mucosal tolerance and modulating the immune response to the β_2GPI.

According to yet another aspect of the present invention there is provided a method for modulating an immune response to an atheroma plaque-related antigen in a subject in need thereof, the method effected by administering to a mucosal surface of the subject a mucosal tolerance-inducing amount of an active ingredient selected from the group consisting of beta$_2$-glycoprotein-1 (β_2GPI) or a derivative of β_2GPI, thereby inducing mucosal tolerance and modulating the immune response to the atherosclerotic plaque antigen.

According to further features in preferred embodiments of the invention described below the β_2GPI is human β_2GPI.

According to yet further features in preferred embodiments of the invention described below, the administering is effected by oral, enteral, buccal, nasal, bronchial, intrapulmonary or intra- peritoneal administration.

According to still further features in preferred embodiments of the invention described below the method further includes administering a therapeutic amount of at least one additional compound selected from the group consisting of HMGCoA reductase inhibitors (statins), mucosal adjuvants, corticosteroids, anti-inflammatory compounds, analgesics, growth factors, toxins, and additional tolerizing antigens.

According to further features in preferred embodiments of the invention described below, modulating is reducing immune reactivity to β_2GPI in the subject.

According to yet further features in preferred embodiments of the invention described below the immune response is selected from the group consisting of Th1 type cytokines expression, Th2 type cytokines expression, and T-cell proliferation.

According to further features in preferred embodiments of the invention described below the atheroma plaque-related antigen is selected from the group consisting of beta$_2$-glycoprotein-1 (β_2GPI), oxidized LDL (oxLDL) and heat shock protein (HSP 60/65).

According to still further features in preferred embodiments of the invention described below, modulating is reducing immune reactivity to the atherosclerotic plaque-related antigen in the subject.

According to an additional aspect of the present invention there is provided an article of manufacture, packaged and identified for use in modulating an immune response to an atherosclerotic plaque antigen in a subject in need thereof. The article of manufacture includes a packaging material and a mucosal tolerance-inducing amount of an active ingredient selected from the group consisting of beta$_2$-glycoprotein-1 (β_2GPI) or a derivative of β_2GPI, and the packaging material includes a label or package insert indicating that the mucosal tolerance-inducing amount of the active ingredient is for modulating an immune response to an atherosclerostic plaque antigen in the subject via mucosal administration.

According to further features in preferred embodiments of the invention described below the atheroma plaque-related antigen is selected from the group

consisting of beta$_2$-glycoprotein-1 (β_2GPI), oxidized LDL (oxLDL) and heat shock protein (HSP 60/65).

According to yet further features in preferred embodiments of the invention described below the immune response is selected from the group consisting of Th1 type cytokines expression, Th2 type cytokines expression, and T-cell proliferation.

According to another aspect of the present invention there is provided an article of manufacture, packaged and identified for use in the prevention and/or treatment of a vascular condition in a subject in need thereof. The article of manufacture includes a packaging material and a mucosal tolerance-inducing amount of an active ingredient selected from the group consisting of beta$_2$-glycoprotein-1 (β_2GPI) or a derivative of β_2GPI, and the packaging material includes a label or package insert indicating that the mucosal tolerance-inducing amount of the active ingredient is for prevention and/or treatment of a vascular condition in the subject via mucosal administration.

According to further features in preferred embodiments of the invention described below the β_2GPI is human β_2GPI.

According to yet further features in preferred embodiments of the invention described below the vascular condition is selected from the group consisting of atherosclerosis, cardiovascular disease, cerebrovascular disease, peripheral vascular disease, stenosis, restenosis and/or in-stent-stenosis.

According to still further features in preferred embodiments of the invention described below the article of manufacture further includes a therapeutically effective amount of at least one additional compound selected from the group consisting of HMGCoA reductase inhibitors (statins), mucosal adjuvants, corticosteroids, anti-inflammatory compounds, analgesics, growth factors, toxins, and additional tolerizing antigens.

BRIEF DESCRIPTION OF THE DRAWINGS

The invention is herein described, by way of example only, with reference to the accompanying drawings. With specific reference now to the drawings in detail, it is stressed that the particulars shown are by way of example and for purposes of illustrative discussion of the preferred embodiments of the present invention only,

and are presented in the cause of providing what is believed to be the most useful and readily understood description of the principles and conceptual aspects of the invention. In this regard, no attempt is made to show structural details of the invention in more detail than is necessary for a fundamental understanding of the invention, the description taken with the drawings making apparent to those skilled in the art how the several forms of the invention may be embodied in practice.

In the drawings:

FIG. 1 illustrates inhibition of early atherogenesis in apo-E deficient mice by nasal tolerance induced by administration of low doses of plaque associated molecules. 9-13 week old apo-E deficient mice were exposed intranasally, with mild sedation, to 3 doses of 10 μg/mouse each HSP 65 (HSP-65)(n=12), human oxidized LDL (H-oxLDL)(n=14), human β_2GPI (β_2GPI)(n=13), bovine serum albumin (BSA) or sham exposure to saline (PBS)(n=12). All mice received the atherogenic "Western" diet following last exposure. Atherogenesis is expressed as the area of atheromatous lesions in the aortic sinus 5 weeks following the 3rd exposure.

FIG. 2 illustrates superior inhibition of early atherogenesis in apo-E deficient mice by mucosal tolerance induced by intranasal exposure to exceedingly low doses of HSP 65. Nasal tolerance was induced in 12-16 week old apo-E deficient mice by intranasal administration of 3 doses of 1μg/mouse HSP65 (HSP-65 low)(n=16) or 10μg/mouse HSP65 (HSP-65 high)(n=14) every other day for 5 days. Control mice were exposed intranasally to an identical volume (10μl) of bovine serum albumin, 10μg/mouse (BSA)(n=14), or sham exposure to PBS (PBS)(n=14). All mice received the atherogenic "Western" diet following last exposure. Atherogenesis is expressed as the area of atheromatous lesions in the aortic sinus 5 weeks after the last nasal exposure.

FIG. 3 illustrates superior suppression of immune reactivity to atheroslerotic plaque antigens induced by nasal exposure to human β_2GPI. 5 week old male apo-E deficient mice were exposed intranasally to 10μg/mouse human β_2GPI (H-β_2-nt)(n=3); or alternately fed, by gavage, with 100μg/mouse human β_2GPI (H-β_2-ot)(n=3) in 0.2 ml PBS; or fed PBS alone (PBS)(n=3) every other day for 5 days. One week following the last feeding the mice were sensitized with a single subcutaneous injection of 10μg/mouse human β_2GPI in 0.1 ml volume. Ten days

later T-cells from inguinal lymph node were prepared as described in Materials and Methods section that follows, and exposed to the sensitizing human β_2GPI antigen for in-vitro assessment of proliferation. Proliferation, indicating immune reactivity, is expressed as the ratio between incorporation of labeled thymidine into the T-cell's DNA in the presence and absence of human β_2GPI antigen (stimulation index, S.I.).

FIG. 4 is a histogram illustrating the inhibition of atherogenesis in LDL RD mice by mucosal administration of β_2GPI. Human (H-β_2GPI) and bovine (B-β_2GPI) (50 or 500μg) β_2GPI, BSA (500μg) or PBS were administered orally (by gavage, as described in the Examples section hereinbelow) to LDL-receptor deficient mice (16–17 mice per group) and a "Western" (atherogenic) diet was then commenced for 4 weeks. Atherosclerotic lesion size (μm^2) was determined at the aortic sinus. *$p < 0.001$ as compared with BSA fed; **$p < 0.0001$ as compared with BSA fed. Note the highly significant inhibition of atherogenesis in all groups receiving β_2GPI (40-50%).

FIGs. 5a-5d are a series of photomicrographs showing representative oil-red O stained sections through the upper sections of the aorta from LDL-RD mice receiving oral administration of β_2GPI (5C- bovine β_2GPI, 50μg/mouse; 5D, human β_2GPI, 50μg/mouse), BSA (5B, 500μg/mouse) or PBS (5A). Upon sacrifice, the hearts and upper aorta were removed from all mice, embedded in OCT medium, frozen and sectioned as described hereinbelow. Note the marked reduction in fatty streak lesions (staining red) in the aorta sections from the β_2GPI treated mice.

FIG. 6 is a histogram demonstrating the inhibition of progression of advanced atherosclerotic plaquing in Apo E KO mice. Human β_2GPI (50μg/mouse, n=16) or PBS (n=16) was administered orally (by gavage) to 20 week old mice, as described hereinbelow. Atherosclerotic lesion size (μm^2) was determined in cryosections of the aortic sinus at 16 weeks from first treatment. Note the > 30% inhibition of atherosclerotic plaque progression from time of initiating treatment (time 0) in the human β_2GPI-fed mice.

FIGs. 7a–7b are histograms showing the inhibition by mucosal administration of β_2GPI of cellular immune responses to atheroma-associated antigens in LDL RD mice. Proliferation of lymph-node cells from mice receiving oral administration of bovine β_2GPI (gray bars) or BSA (hatched bars) was assessed in vitro by thymidine

uptake in the presence of different concentrations of β_2GPI (7A). Proliferation of lymph-node cells from mice immunized against oxLDL in addition to receiving oral administration of bovine β_2GPI (gray bars) or BSA (hatched bars) was assessed in vitro by thymidine uptake in the presence of different concentrations of oxLDL (7B). Thymidine uptake is expressed as the Stimulation Index. Note the marked suppression of cellular immune response to both β_2GPI and oxLDL stimulation conferred by oral administration of β_2GPI. *$p < 0.05$.

FIG. 8 is a histogram showing the induction of anti-inflammatory Th2 cytokines by oral administration of β_2GPI. Conditioned medium was collected from lymph node cells of mice orally tolerized with β_2GPI (hatched bars) or BSA (solid bars), immunized with β_2GPI and incubated with β_2GPI (10μg/ml) for 48 h. Levels of IL-4 and IL-10 were detected in the medium employing a capture ELISA kit as described in the Examples section hereinbelow. Note the remarkable increase in anti-inflammatory cytokines (IL-10 and IL-4) in cells from β_2GPI-tolerized mice. *$p < 0.01$.

FIG. 9 is a photograph of RT-PCR products demonstrating the induction of an anti-inflammatory response in aortic tissue from mice tolerized with β_2GPI. Aorta tissue from 7-9 week old ApoE KO mice receiving oral administration (by gavage, as described hereinbelow) of β_2GPI (100μg) or PBS, every other day for 10 days was removed, and RNA was prepared as described. Expression of cytokines IL-10 and IFN-γ, and the housekeeping gene β-actin, was measured by RT-PCR, using specific oligo primers. The upper panel is a photograph of the ethidium bromide stained gel of PCR products showing the induction of anti-inflammatory IL-10 expression in the β_2GPI-tolerized mice. The middle panel is a photograph of the ethidium bromide stained gel of PCR products showing the suppression of pro-inflammatory IFN-γ transcription in the β_2GPI-tolerized mice. Note the lack of effect of mucosal administration of β_2GPI on overall transcription rate, as evidenced by the unchanged levels of β-actin transcription (lower panel).

DESCRIPTION OF THE PREFERRED EMBODIMENTS

The present invention is of methods and compositions employing beta$_2$-glycoprotein-1 (β_2GPI) or derivatives of β_2GPI effective in inducing mucosal tolerance to atheroma related antigens, thus inhibiting inflammatory processes contributing to atheromatous vascular disease and sequalae.

The principles and operation of the present invention may be better understood with reference to the drawings and accompanying descriptions.

Before explaining at least one embodiment of the invention in detail, it is to be understood that the invention is not limited in its application to the details set forth in the following description or exemplified by the Examples. The invention is capable of other embodiments or of being practiced or carried out in various ways. Also, it is to be understood that the phraseology and terminology employed herein is for the purpose of description and should not be regarded as limiting.

Experimental and clinical evidence indicates a causative role for atheroma plaque-associated antigens in the etiology of the excessive inflammatory response in atherosclerosis. Both cellular and humoral immune reactivity to the plaque associated molecules oxidized LDL, β_2GPI and HSP 65 have been demonstrated, suggesting an important anti-oxidized LDL auto-immune component in atherogenesis. Thus, oxidized LDL, β_2GPI and HSP 65, and components thereof, have been the targets of numerous therapies for prevention and treatment of heart disease, cerebral-vascular disease and peripheral vascular disease.

Prior art teaches the application of plaque-associated antigens for detection and diagnosis of atherosclerosis and other plaque- and thrombosis related conditions. For example, Holvoet (US Pat No. 6,309,888) teaches the use of stage specific plaque associated antigens oxLDL and MDA-LDL for screening for Coronary Artery disease. Similarly, others (US Pat Nos. 5,472, 883, 5,506,110, 5,900,359, and 5,998,223 and US Patent Application No. 10/488688 to Matsuura, et al, US Patent No: 5,344,758 to Krilis, et al, US Patent No: 5,750,309 to Wilson et al, US Patent Application No: 10/492479 to Koike et al, and Iverson et al., Immunology 1998;95:15542-46) have disclosed the use of anti β_2GPI antibodies, to screen for serum indicators of APLS, SLE, cerebral infarct and atherosclerosis. The

abovementioned disclosures propose diagnostic applications alone, and fail to recognize the therapeutic potential of these plaque associated molecules.

Although the role of immune response in the etiology and progression of atherosclerosis and other plaque related diseases remains controversial (see Meir, K, et al, International Atherosclerosis Soc. 2001 Commentary), many immune-based therapies have been proposed for atherosclerosis. General methods of reducing immune response in inflammatory and hyperreactive conditions are taught in, for example US Patent Nos 6,277,969; 5,698,195 and 5,656,272 to Le at al, and 6,224,902 to Alving, et al, International Patent Application Nos. 001 001 2514 to Shurkovitz et al and 20010051156 A1 to Zeng. However, the proposed reduction or removal of mediators of immune reactivity, such as cytokines, tumor necrosis factor (TNF) and other pathogenic factors requires ongoing costly and potentially dangerous methods such as immunoadsorption of blood and prolonged anti-cytokine administration. Furthermore, no application to treatment of atherosclerosis or plaque-related disease is disclosed.

Specific immunotherapy with atheroma plaque-associated antigens has also been proposed. Bumol, et al, Calenoff, et al and Takano, et al (US Pat Nos. 5,196,324; 6,025,477 and 5,110,738, respectively) disclose the use of crude, poorly defined fractionated plaque preparations for immunization, monoclonal Ab preparation, diagnosis and treatment of atherosclerosis. These antigens, protein and lipid fractions of atheromatous tissue, are poorly defined, impractical for therapeutic use, and potentially hazardous in prolonged treatment.

Prior art teaches immunotherapy directed against atheroma-associated antigens. Zhou, et al (Arterioscler Thromb Vasc Biol, 2001;21:108) achieved a significant reduction in early plaque formation in mice following footpad immunization with homogenized plaque or homologous MDA LDL. Palinski et al (PNAS USA 1995;92:821-25) produced similar levels of protection in rabbits immunized with oxidized LDL. However, application of conventional immunization techniques to atheroma plaque components is problematic, since the adjuvant preparations required for immunization and boosters have produced accelerated plaque formation in similar regimen of immunization. Furthermore, relatively high

doses (100 μgram/ mouse/ injection) of plaque antigen were required for immunity. Mucosal administration and induction of tolerance were not mentioned.

Recent animal and in-vitro studies with β_2GPI and other components of anticardiolipin and antiphospholipid antigens (see George J, et al Rheum Dis Clin North Am 2001;27:603-10; Brey, et al Stroke 2001;32:1701-06; Kyobashi, et al J Lipid Res 2001;42:697-709; Koike T, et al Ann Med 2000;32,Suppl. I:27-31, Cabral AR et al Am J Med 1996;101:472-81, Bili et al. Circulation 2000; 102:1258-; Altman, R. Thrombosis Journal 2003;1:4, pgs 1-11; Segovia, J of Rheumatology; Hatori et al. Arthritis Rheum. 2000;43:65-75; and Peirangeli et al J Autoimmunity 2004;22:217-25) have demonstrated the association of β_2GPI with antiphospholipid syndrome, thrombosis, stroke, APLS, atherosclerosis and myocardial infarction. Although cryptic epitopes of the protein were implicated in humoral and cellular immune response, none of the abovementioned studies demonstrated protective immunity with the protein. Similarly, studies with HSP 65 (Birnie DH Eur Heart J 1998;19:366-67; Xu Q, et al Circulation 1999;100:1169-74; and Gromadzka G, et al Cerebrovasc Dis 2001;12:235-39) have implicated this plaque associated antigen in stroke and heart disease, suggesting that humoral immunity may be a triggering factor.

However, numerous studies which fail to demonstrate a correlation between titers of anti- β_2GPI antibodies and recurrent or other cardiac disorders (see, for example, Bili, et al. Circulation, 2000;102:1258-; Limaye et al Aust and New Zeal J of Med, 1999;29; Levine et al JAMA, 2004;291:576-84; Erkkila et al Atherosclerosis 2000; 20:204-9; Manzi, Rheumatology 2000;39:353-359; Sadovsky, Amer Fam Phys Dec 1999) confound the understanding of the association of β_2GPI with vascular and cardiac events.

The complexity of atheroma plaque antigen immunity in atherosclerosis was demonstrated by Schoenfeld Y, et al (Autoimmunity 2000;15:199-202), and George et al (Circulation, 1998;98:1108-1115), who immunized LDL-receptor deficient (KO) mice with both β_2GPI and HSP 65 protein antigens, producing strong cellular and humoral responses, and surprisingly enhanced plaque formation. Similar increased atherogenesis was observed with passive transfer of β_2GPI activated lymphocytes (George et al. Circulation 2000;102:1822-27). None of the above

mentioned studies demonstrated inhibition of atherogenic processes by immune tolerance.

Suppression of immune response to autoantigens in atherosclerosis and related disease has been recently investigated. Victoria et al (US Patent Nos. 6,410,775, 6,207,160 and 5,844,409), Coutts et al (US Patent Application No. 10/081076), disclose specific non-immunogenic β_2GPI peptides lacking T cell epitopes for reducing antibody binding of immune cells and inducing B-cell tolerance in APLS, SLE and other diseases. However, no actual protection was demonstrated, and the disclosures emphasize the diagnostic use of the non immunogenic peptides. George J, et al (Atherosclerosis 1998;138:147-52) has demonstrated the feasibility of immune suppression by hyperimmunization with MDA LDL and reduction of atherogenesis in mice. However, impractically large doses of antigen were required, and the paradoxical response to immunization with plaque antigens obviates the clinical efficacy of such therapy. Furthermore, none of the abovementioned studies disclose induction of mucosal tolerance for treatment of atherosclerosis.

Oral and mucosal tolerance for suppression and prevention of inflammatory conditions is well known in the art. For example, Weiner et al. have disclosed therapy, for the treatment of rheumatoid arthritis by mucosal administration of collagen and collagen peptides (US Patent Nos. 5,399,347; 5,720,955; 5,733,542; 5,843,445; 5,856,446; and 6,019,975), treatment of Type I diabetes by mucosal administration of insulin (5,643,868; 5,763,396; 5,843,445; 5,858,968; 6,645,504; and 6,703,361) or glucagon (6,645,504), uveoretinitis by mucosal administration of toleragens (5,961,977), and multiple sclerosis by mucosal administration of myelin basic protein (MBP) (5,849,298; 5,858,364; 5,858,980; 5,869,093; 6,077,509). Additional candidate conditions, antigens and modes of treatment by mucosal tolerance have been disclosed in US Patent Nos. 6,812,205, 5,935,577; 5,397,771; 4,690,683 to Weiner et al., US Patent No. 6,790,447 to Wildner et al; International Patent Nos. EP 0886471 A1, WO 01821951 to Haas, et al, US Patent No. 5,843,449 to Boots et al. (HCgp-39 for arthritis), and US Patent Application No: 10/437404 to Das (mucosal tolerance and relief from Crohn's disease by administration of Colonic Epithelial Protein).

US Pat Application No 09/806,400 to Shoenfeld et al filed Sept 30, 1999, which is incorporated herein in its entirety, teaches the oral administration of plaque associated antigens for the induction of tolerance in LDL receptor deficient mice. Measuring arterial fatty streak lesion density, the inventors demonstrated that oral administration of oxidized LDL, β_2GPI and HSP 65 derived from animal sources were each able to produce approximately 30% reduction in atherogenesis. Additional evidence for the efficacy of mucosal tolerance with atheroma-associated antigen is provided in U.S. patent application Nos. 10/989,724, filed November 17, 2004, 10/451,370, filed July 2, 2003, 09/944,592, filed September 4, 2001, and U.S. patent application No. 09/806,400, filed March 30, 2001, (which are incorporated herein by reference, as if fully set forth).

While reducing the present invention to practice, the present inventors have uncovered that mucosal administration of β_2GPI results in the induction of mucosal tolerance, suppression of anti- β_2GPI related immune reactivity and protection from atherosclerosis. Mucosal tolerance according to the invention is an advantageous method for treating vascular disorders for several reasons:

(1) Absence of toxicity: no toxicity has been observed in clinical trials or animal experiments involving oral or other mucosal administration of protein antigens, such as bovine myelin [which contains myelin basic protein (MBP) and proteolipid protein (PLP)] to humans afflicted with multiple sclerosis, or oral or by-inhalation administration of chicken Type II collagen to humans or rodents afflicted with rheumatoid arthritis [or a corresponding animal model disorder]; or oral administration of bovine S-antigen to humans afflicted with uveoretinitis; or oral administration of insulin to healthy volunteers.

(2) Containment of immunosuppression. Conventional treatments of immune system disorders involve administration of non-specific immunosuppressive agents, such as the cytotoxic drugs methotrexate, cyclophosphamide (CYTOXAN.RTM., Bristol-Myers Squibb), azathioprine (IMURAN.RTM., Glaxo Wellcome) and cyclosporin A (SANDIMMUNE.RTM., NEORAL.RTM., Novartis). Steroid compounds such as prednisone and methylprednisolone (also non-specific immunosuppressants) are also employed in many instances. All of these currently employed drugs have limited efficacy (e.g., against both cell-mediated and antibody-

mediated autoimmune disorders). Furthermore, such drugs have significant toxic and other side effects and, more important, eventually induce "global" immunosuppression in the subject being treated. Prolonged treatment with the drugs down-regulates the normal protective immune response against pathogens, thereby increasing the risk of infection. In addition, patients subjected to prolonged global immunosuppression have an increased risk of developing severe medical complications from the treatment such as malignancies, kidney failure and diabetes.

(3) Convenience of therapy. Mucosal administration is more convenient than parenteral, or other forms, of administration.

(4) Greatly reduced incidence of alteration of the tolerizing molecule by digestive and metabolic processes (especially in non-oral routes of administration). These advantages provide superior protection from atherogenic processes, improved patient compliance and reduced cost of therapy.

Thus, according to one aspect of the present invention there is provided a method for prevention and/or treatment of a vascular condition in a subject in need thereof. The method, according to this aspect of the present invention is effected by administering to a mucosal surface of a subject (e.g., a human) a mucosal tolerance-inducing amount of an antigenic portion of an active ingredient selected from the group consisting of beta$_2$-glycoprotein-1 (β_2GPI) or a derivative of β_2GPI, thereby inducing mucosal tolerance.

As used herein, the phrase "mucosal surface" is defined as a portion of the anatomy having exposed mucosal membranes having component or components of the mucosal associated lymphatic tissue. As used herein, the phrase "mucosal administration" is defined as application of any and all compounds and/or compositions to at least one mucosal surface. Non-limiting examples of mucosal administration are buccal, intranasal, otic (middle ear), conjunctival, vaginal, rectal, etc. Mucosal administration excludes, for example, intravenous, subcutaneous and epidural administration.

In preferred embodiments of the present invention, mucosal tolerance is effected by administering to a mucosal surface of the subject a mucosal tolerance-inducing amount of an active ingredient selected from the group consisting of beta$_2$-glycoprotein-1 (β_2GPI) or a derivative of β_2GPI. β_2GPI proteins have been identified

in many phylogenetically diverse species, and β_2GPI protein suitable for use in the present invention includes, but is not limited to, the following β_2GPI (also known as Apolipoprotein H, Apo-H, Activated protein C-binding protein, APC inhibitor, AntiCL cofactor) amino acid sequences:

Human β_2GPI precursor- GenBank Accession No.P02749, Human Apo-H precursor- GenBank Accession No. NP000033 (SEQ ID NO: 10), NBHU, Canis familiaris Apo-H GenBank Accession No. NP001002858, precursor GenBank Accession No. JN0465, Bos Taurus Apo-H- GenBank Accession No. NP776417, precursor GenBank Accession No. NBBO, Mus musculus Apo-H GenBank Accession No. NP038503, CAA72190; precursor GenBank Accession No. NBMS, Rattus norvegicus Apo-H precursor GenBank Accession No. NBRT, β_2GPI precursor Human GenBank Accession No. AAH26283, β_2GPI precursor Human GenBank Accession No. AAH20703, β_2GPI precursor Pan troglodytes GenBank Accession No. Q95LB0, β_2GPI precursor bovine GenBank Accession No. P17690, β_2GPI precursor Rat, GenBank Accession No. P26644, β_2GPI precursor Canis fam. GenBank Accession No. P33703, β_2GPI precursor Mus musc. GenBank Accession No. Q01339, β_2GPI Mus musc GenBank Accession No. BAA00945, CAA69701, AAB30789, β_2GPI Apo H Human GenBank Accession No. AAP72014, β_2GPI Human GenBank Accession No. CAA76845, CAA72279, CAA37664, CAA40977, CAA41113, AAB21330 , and β_2GPI bovine GenBank Accession No. CAA42669. In a further preferred embodiment, the β_2GPI is human β_2GPI (SEQ ID NO: 10).

As used herein, a "derivative" of a protein peptide is a polypeptide which contains one or more modifications to the primary amino acid sequence of a protein peptide. Accordingly, a derivative of a β_2GPI protein peptide is a polypeptide which contains one or more modifications to the primary amino acid sequence of an β_2GPI protein peptide. Modifications which create a β_2GPI derivative can be made to an β_2GPI protein peptide for a variety of reasons, including 1) to reduce or eliminate an activity of a β_2GPI protein peptide; 2) to enhance a property of a β_2GPI protein peptide; 3) to provide a novel activity or property to a β_2GPI protein peptide; or 4) to establish that an amino acid substitution that does or does not affect β_2GPI protein peptide activity. Modifications to a β_2GPI protein peptide are typically made to the

nucleic acid which encodes the β_2GPI protein peptide, and can include deletions, point mutations, truncations, amino acid substitutions and additions of amino acids or non-amino acid moieties. Alternatively, modifications can be made directly to the protein peptide, such as by cleavage, addition of a linker molecule, addition of a detectable moiety (for example, biotin, fluorophore, radioisotope, enzyme, or peptide), addition of a fatty acid, and the like.

Modifications also embrace fusion proteins comprising all or part of the β_2GPI amino acid sequence. One of skill in the art will be familiar with methods for predicting the effect on protein conformation of a change in protein sequence, and can thus "design" a β_2GPI derivative according to known methods. One example of such a method is described by Dahiyat and Mayo in Science 278:82-87 (1997), whereby proteins can be designed de novo. The method can be applied to a known protein to vary only a portion of the polypeptide sequence. By applying the computational methods of Dahiyat and Mayo, specific derivatives of a β_2GPI protein peptide can be proposed and tested to determine whether the derivative retains a desired conformation.

β_2GPI derivatives include β_2GPI protein peptides which are modified specifically to alter a feature of the polypeptide unrelated to its physiological activity. For example, cysteine residues can be substituted or deleted to prevent unwanted disulfide linkages. Similarly, certain amino acids can be changed to enhance expression of a β_2GPI protein peptide by eliminating proteolysis by proteases in an expression system (e.g., dibasic amino acid residues in yeast expression systems in which KEX2 protease activity is present).

Mutations of a nucleic acid which encode a β_2GPI protein peptide preferably preserve the amino acid reading frame of the coding sequence, and preferably do not create regions in the nucleic acid which are likely to hybridize to form secondary structures, such as hairpins or loops, which can be deleterious to expression of the derivative polypeptide.

Mutations can be made by selecting an amino acid substitution, or by random mutagenesis of a selected site in a nucleic acid which encodes the polypeptide. Derivative polypeptides are then expressed and tested for one or more activities to determine which mutation provides a derivative polypeptide with a desired property.

Further mutations can be made to derivative (or to native β_2GPI protein peptides) which are silent as to the amino acid sequence of the polypeptide, but which provide preferred codons for translation in a particular host. The preferred codons for translation of a nucleic acid in, e.g., E. coli, are well known to those of ordinary skill in the art. Still other mutations can be made to the noncoding sequences of a β_2GPI gene or cDNA clone to enhance expression of the polypeptide.

The activity of derivatives of β_2GPI protein peptides can be tested by cloning the gene encoding the derivative or variant β_2GPI protein peptide into a prokaryotic or eukaryotic (e.g., mammalian) expression vector, introducing the vector into an appropriate host cell, expressing the derivative β_2GPI protein peptide, and testing for a functional capability of the β_2GPI protein peptides as disclosed herein. For example, the derivative β_2GPI protein peptide can be tested for its ability to bind to an anti- β_2GPI antibody, to elicit an immune response in a sensitized animal, or to suppress a vascular disorder, as set forth below in the examples.

The skilled artisan will also realize that conservative amino acid substitutions may be made in β_2GPI protein peptides to provide functionally equivalent derivatives (functional equivalents) of the foregoing polypeptides, i.e., derivatives which retain the functional capabilities of the β_2GPI protein peptides. As used herein, a "conservative amino acid substitution" refers to an amino acid substitution which does not alter the relative charge or size characteristics of the polypeptide in which the amino acid substitution is made. Derivatives can be prepared according to methods for altering polypeptide sequence known to one of ordinary skill in the art such as are found in references which compile such methods, e.g., Molecular Cloning. A Laboratory Manual, J. Sambrook, et al., eds., Second Edition, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., 1989, or Current Protocols in Molecular Biology, F M Ausubel, et al., eds., John Wiley & Sons, Inc., New York. Exemplary functionally equivalent derivatives of the β_2GPI protein peptides include polypeptides having conservative amino acid substitutions of SEQ ID NO.10(Human beta2GPI). Conservative substitutions of amino acids include substitutions made amongst amino acids within the following groups: (a) M, I, L, V; (b) F, Y, W; (c) K, R, H; (d) A, G; (e) S, T; (f) Q, N; and (g) E, D.

As used herein, the phrase "mucosal tolerance-inducing amount" of β_2GPI or a derivative of β_2GPI is defined as the amount sufficient to stimulate a reduction in immune reactivity to β_2GPI, which reduction in immune reactivity can also be associated with decreased stimulation index of lymph node cells, decreased cytokine production, inhibition of atherogenic processes in the recipient thereof, and the like, assessment of which is described in detail in the Examples section hereinbelow.

As used herein, the phrases "atheroma plaque related antigens" or "atheroma plaque-associated antigens" are defined as any and all protein, carbohydrate, lipid and nucleic acid molecules, portions thereof (antigenic portions), their derivatives, or combinations thereof physically or functionally related to the etiology, pathogenesis, symptomatology and/or treatment of a plaque-related condition or disease. Such molecules may be, for example, plaque components such as oxidized LDL, foam cell components, etc, but may also include humoral and cellular entities, such as antibodies, cytokines, growth factors and T cell receptors.

As used herein, the phrase "antigen" refers to a portion of a molecule capable of eliciting an immune response. For example, in cases where the molecule is a protein (e.g. β_2GPI) such a portion can include a stretch of 6-8 amino acids that constitute an antigenic epitope. Methods for predicting antigenic portions are well known in the art, for example, DNASTAR'S PROTEAN sequence analysis and prediction module (DNAStar, Madison, WI). As such determining antigenic portions of plaque-associated molecules suitable for use with the present invention is well within the capabilities of an ordinarily skilled artisan.

β_2GPI (as well as fragments, analogs, portions and derivatives thereof) can be purified from natural sources (the tissue or organ where β_2GPI normally occurs) and can also be obtained using recombinant DNA technology, in bacterial, yeast, insect (e.g. baculovirus) and mammalian cells using techniques well-known to those of ordinary skill in the art.

As used herein the term "vascular disorder" refers to a disease or process involving tissue intrinsic to the blood vessels, particularly the arterial vessels, in which the lumen of affected vessels are narrowed as a result. The archetype of vascular disorder is atherosclerosis. A vascular disorder can involve vessels associated with one or more vascular beds, e.g., the coronary arteries, the cerebral

arteries, the aorta, the renal arteries, the splanchnic bed, the peripheral arteries, etc. Included are arterial aneurysms, e.g., aortic aneurysm. Such aneurysms are preferably non-traumatic in origin and can but need not necessarily be atherosclerotic. Also included are a number of principally inflammatory vascular disorders, including but not limited to: allergic angiitis and granulomatosis (Churg-Strauss disease), Behget's syndrome, Cogan's syndrome, graft-versus-host disease (GvHD), Henoch-Schonlein purpura, Kawaski disease, leukocytoclastic vasculitis, polyarteritis nodosa (PAN), microscopic polyangiitis, polyangiitis overlap syndrome, Takayasu's arteritis, temporal arteritis, transplant rejection, Wegener's granulomatosis, and thromboangiitis obliterans (Buerger's disease).

Immune tolerance established using the present methodology can be used in the prevention and/or treatment of disorders associated with plaque formation, including but not limited to atherosclerosis, atherosclerotic cardiovascular disease, cerebrovascular disease, peripheral vascular disease, stenosis, restenosis and in-stent-stenosis. Some non-limiting examples of atherosclerotic cardiovascular disease are myocardial infarction, coronary arterial disease, acute coronary syndromes, congestive heart failure, angina pectoris and myocardial ischemia. Some non-limiting examples of peripheral vascular disease are gangrene, diabetic vasculopathy, ischemic bowel disease, thrombosis, diabetic retinopathy and diabetic nephropathy. Non-limiting examples of cerebrovascular disease are stroke, cerebrovascular inflammation, cerebral hemorrhage and vertebral arterial insufficiency. Stenosis is occlusive disease of the vasculature, commonly caused by atheromatous plaque and enhanced platelet activity, most critically affecting the coronary vasculature. Restenosis is the progressive re-occlusion often following reduction of occlusions in stenotic vasculature. In cases where patency of the vasculature requires the mechanical support of a stent, in-stent-stenosis may occur, re-occluding the treated vessel. The measurable symptoms and diagnostic markers of these vascular disorders are well established in the literature and known to physicians practicing in this field. See, for example, Harrison's Principles of Internal Medicine, 14th ed., A S Fauci et al., eds., New York: McGraw-Hill, 1998.

The methods of the present invention can be administered as a sole therapeutic and/or preventive treatment, or in conjunction with one or more

additional treatments. Conventional treatment modalities for atherosclerosis, and other vascular conditions include, but are not limited to various anti-inflammatory, analgesic, and anti-coagulant agents well known in the art. Thus, according to a preferred embodiment of the present invention the β_2GPI or derivative thereof is administered along with at least one additional compound selected from the group consisting of HMGCoA reductase inhibitors (statins), mucosal adjuvants, corticosteroids, anti-inflammatory compounds, analgesics, growth factors, toxins and additional tolerizing antigens. It will be appreciated by one of ordinary skill in the art, that the additional compounds or treatment regimen are administered in a conventional manner, and not to mucosal surfaces in a manner so as to induce mucosal tolerance thereto. In addition, it will be appreciated that use of the methods of the present invention does not preclude the initiation or continuation of other therapies for the abovementioned diseases or conditions, except where specifically counter-indicated.

HMGCoA reductase inhibitors that can be administered in combination with β_2GPI or derivative thereof include, but are not limited to, Pravastatin (PRAVACHOL, Bristol-Myers Squibb), Lovastatin (MEVACOR, Merck), Simvastatin (ZOCOR, Merck), Fluvastatin (LESCOL, Novartis), Atorvastatin (LIPITOR, Parke-Davis), Cerivastatin (BAYCOL, Bayer), Rosuvastatin (CRESTOR, Astra-Zeneca) and Lovastatin + extended release niacin (ADVICOR, Kos Pharmaceutical).

Anti-inflammatory drugs that can be administered in combination with the β_2GPI or derivative thereof of the present invention include, but are not limited to, Alclofenac; Alclometasone Dipropionate; Algestone Acetonide; Alpha Amylase; Amcinafal; Amcinafide; Amfenac Sodium; Amiprilose Hydrochloride; Anakinra; Anirolac; Anitrazafen; Apazone; Balsalazide Disodium; Bendazac; Benoxaprofen; Benzydamine Hydrochloride; Bromelains; Broperamole; Budesonide; Carprofen; Cicloprofen; Cintazone; Cliprofen; Clobetasol Propionate; Clobetasone Butyrate; Clopirac; Cloticasone Propionate; Cormethasone Acetate; Cortodoxone; Deflazacort; Desonide; Desoximetasone; Dexamethasone Dipropionate; Diclofenac Potassium; Diclofenac Sodium; Diflorasone Diacetate; Diflumidone Sodium; Diflunisal; Difluprednate; Diftalone; Dimethyl Sulfoxide; Drocinonide; Endrysone; Enlimomab;

Enolicam Sodium; Epirizole; Etodolac; Etofenamate; Felbinac; Fenamole; Fenbufen; Fenclofenac; Fenclorac; Fendosal; Fenpipalone; Fentiazac; Flazalone; Fluazacort; Flufenamic Acid; Flumizole; Flunisolide Acetate; Flunixin; Flunixin Meglumine; Fluocortin Butyl; Fluorometholone Acetate; Fluquazone; Flurbiprofen; Fluretofen; Fluticasone Propionate; Furaprofen; Furobufen; Halcinonide; Halobetasol Propionate; Halopredone Acetate; Ibufenac; Ibuprofen; Ibuprofen Aluminum; Ibuprofen Piconol; Ilonidap; Indomethacin; Indomethacin Sodium; Indoprofen; Indoxole; Intrazole; Isoflupredone Acetate; Isoxepac; Isoxicam; Ketoprofen; Lofemizole Hydrochloride; Lomoxicam; Loteprednol Etabonate; Meclofenamate Sodium; Meclofenamic Acid; Meclorisone Dibutyrate; Mefenamic Acid; Mesalamine; Meseclazone; Methylprednisolone Suleptanate; Momiflumate; Nabumetone; Naproxen; Naproxen Sodium; Naproxol; Nimazone; Olsalazine Sodium; Orgotein; Orpanoxin; Oxaprozin; Oxyphenbutazone; Paranyline Hydrochloride; Pentosan Polysulfate Sodium; Phenbutazone Sodium Glycerate; Pirfenidone; Piroxicam; Piroxicam Cinnamate; Piroxicam Olamine; Pirprofen; Prednazate; Prifelone; Prodolic Acid; Proquazone; Proxazole; Proxazole Citrate; Rimexolone; Romazarit; Salcolex; Salnacedin; Salsalate; Sanguinarium Chloride; Seclazone; Sermetacin; Sudoxicam; Sulindac; Suprofen; Talmetacin; Talniflumate; Talosalate; Tebufelone; Tenidap; Tenidap Sodium; Tenoxicam; Tesicam; Tesimide; Tetrydamine; Tiopinac; Tixocortol Pivalate; Tolmetin; Tolmetin Sodium; Triclonide; Triflumidate; Zidometacin; Zomepirac Sodium.

Analgesics that can be administered in combination with the β_2GPI or derivative thereof of the present invention include, but are not limited to, acetaminophen, salicylates, butalbital, narcotic analgesics such as fentynal and central analgesics such as tramadol.

Mucosal adjuvants that can be administered in combination with β_2GPI or derivative thereof are well known in the art (see, for example, US Patent Nos. 6,270,758 to Staats, et al and 5,681,571 to Holmgren et al.).

According to another preferred embodiment of the present invention, a combination of at least two of the abovementioned molecules is administered to the subject.

The method of the invention may be used for prevention and/or treatment of non-atherosclerosis related diseases. For example, β_2GPI or a derivative thereof, anti-β_2GPI antibodies, and β_2GPI in complex with phospholipids and phospholipid metabolites have been clearly implicated in the pathogenesis, and therefore potential treatment of additional, non-atherosclerosis-related diseases. Such diseases and syndromes include Anti Phospholipid Syndrome (APLS or APS) (Koike T, et al Ann Med 2000;32 Suppl I:27-31), thrombosis, preeclampsia, acute otitis media, venous sinus thrombosis (Oestricher-Kedem et al. Laryngoscope 2004;114:90-94), scleroderma (Sato et al. Ann Rheum Dis 2003;62:771-74), atopic disease (Ambrozic et al Int Immunology 2002;14:823-30), Systemic Lupus Erythematosus (SLE) (Davies, Rheumatology 2002;41:395-400, and US Pat Nos. 5,344,758 and 6,207,160, to Krilis, et al and Victoria, et al, respectively), venous and arterial thromboses (Cabral AR, et al Am J Med 1996;101:472-81) and others.

While reducing the present invention to practice, it was uncovered that mucosal administration of β_2GPI to LDL-RD mice reduces the T-cell response to stimulation by β_2GPI in previously sensitized animals. Examples 3 and 6 hereinbelow describe the reduction by up to 70% of the stimulation index of lymph node cells (Figs 3 and 7a-b), the induction of anti-inflammatory cytokines IL-10 and IL-4 (Figs 8 and 9), and the suppression of plaque-associated pro-inflammatory INF-γ cytokine expression (Fig. 9).

Thus, the methods of the present invention can be used alter an immune response to β_2GPI in a subject in need thereof. Thus, according to one aspect of the present invention there is provided a method for modulating an immune response to a β_2GPI in a subject in need thereof. The method, according to this aspect of the present invention is effected by administering to a mucosal surface of a subject (e.g., a human) a mucosal tolerance-inducing amount of an antigenic portion of an active ingredient selected from the group consisting of beta$_2$-glycoprotein-1 (β_2GPI) or a derivative of β_2GPI, thereby inducing mucosal tolerance and modulating the immune response to the β_2GPI.

In one preferred embodiment, the subject is at risk for, or suffering from a condition characterized by excess reactivity to β_2GPI, and the modulating is reducing the immune reactivity to β_2GPI in the subject. Both humoral and cellular immune

reactivity can be readily assessed *in-vitro* and *in-vivo*, by one of ordinary skill in the art, according to art-recognized criteria, such as measurement of circulating antibodies, isotype antibodies, cytokine profile, stimulation index, and the like. Thus, according to a preferred embodiment, the immune response is selected from the group consisting of Th2 cytokine expression, Th1 cytokines expression, and T-cell proliferation. Exemplary methods for assessing immune reactivity are described in detail hereinbelow.

Further, while reducing the present invention to practice, it was unexpectedly uncovered that mucosal administration of β_2GPI to LDL-RD mice inhibits not only the T-cell response to stimulation by β_2GPI in sensitized animals, but also the primary immune response to stimulation by other atheroma plaque-associated antigens, such as oxidized LDL (see Figs 7a and 7b) in sensitized animals. Without wishing to be limited by a single hypothesis, this tolerizing effect on oxLDL responsiveness can be mediated through the "bystander effect", involving regulatory cells secreting nonantigen-specific cytokines that suppress inflammation in the microenvironment where the mucosally administered antigen is localized. Thus, according to another aspect of the present invention, there is provided a method for modulating an immune response to an atheroma plaque-related antigen in a subject in need thereof. The method, according to this aspect of the present invention is effected by administering to a mucosal surface of a subject (e.g., a human) a mucosal tolerance-inducing amount of an antigenic portion of an active ingredient selected from the group consisting of beta$_2$-glycoprotein-1 (β_2GPI) or a derivative of β_2GPI, thereby inducing mucosal tolerance and modulating the immune response to the atheroma plaque-related antigen.

Atheroma plaque-related antigens are defined hereinabove. In one preferred embodiment, the atheroma plaque related antigens are selected from the group consisting of β_2GPI, oxidized LDL, and heat-shock protein (HS 60/65).

Suitable formulations according to the invention include formulations adapted for oral, enteral, buccal, nasal, bronchial or intrapulmonary administration. The preparation of such formulations is well within the skill of the art. It is preferred that such formulations not contain substances that can act as adjuvants in order to avoid sensitization of the treated subject. It is also preferred that the antigens employed be

of synthetic provenance and not isolated from biological sources to avoid the risk of infection (notably, but not exclusively, to avoid transmission of agent responsible for the Creutzfeld-Jacob disease). Additionally, it is preferred that the formulation not contain adsorption promoting agents or ingredients that protect against proteolytic degradation.

Suitable oral formulations for use in tolerization of T-cell mediated immune responses according to the present invention can be in any suitable orally administrable form, for example, a pill, a liquid, or a capsule or caplet containing an effective amount of antigen. Each oral formulation may additionally comprise inert constituents including pharmaceutically acceptable carriers, diluents, fillers, disintegrants, flavorings, stabilizers, preservatives, solubilizing or emulsifying agents and salts as is well-known in the art. For example, tablets may be formulated in accordance with conventional procedures employing solid carriers and other excipients well-known in the art. Capsules may be made from any is 1 cellulose derivatives. Nonlimiting examples of solid carriers include starch, sugar, bentonite, silica and other commonly used inert ingredients. Diluents for liquid oral formulations can include inter alia saline, syrup, dextrose and water.

The antigens (i.e., β2GPI and therapeutically effective fragments and analogs thereof) used in the present invention can also be made up in liquid formulations or dosage forms such as, for example, suspensions or solutions in a physiologically acceptable aqueous liquid medium. Such liquid media include water, or suitable beverages, such as fruit juice or tea which will be convenient for the patient to sip at spaced apart intervals throughout the day. When given orally in liquid formulations the antigen may be dissolved or suspended in a physiologically acceptable liquid medium, and for this purpose the antigen may be solubilized by manipulation of its molecule (e.g., hydrolysis, partial hydrolysis or trypsinization) or adjustment of the pH within physiologically acceptable limits (e.g., 3.5 to 8). Alternatively, the antigen may be reduced to micronized form and suspended in a physiologically acceptable liquid medium, or in a solution.

Sustained release oral delivery systems are also contemplated and are preferred. Nonlimiting examples of sustained release oral dosage forms include those described in U.S. Pat. No. 4,704,295, issued Nov. 3, 1987; U.S. Pat. No.

4,556,552, issued Dec. 3, 1985; U.S. Pat. No. 4,309,404, issued Jan. 5, 1982; U.S. Pat. No. 4,309,406, issued Jan. 5, 1982; U.S. Pat. No. 5,405,619, issued Apr. 10, 1995; PCT International Application WO 85/02092, published May 23, 1985; U.S. Pat. No. 5,416,071, issued May 16, 1995; U.S. Pat. No. 5,371,109, issued Dec. 6, 1994; U.S. Pat. No. 5,356,635, issued Oct. 18, 1994; U.S. Pat. No. 5,236,704, issued Aug. 17, 1993; U.S. Pat. No. 5,151,272, issued Sep. 29, 1992; U.S. Pat. No. 4,985,253, issued Jan. 15, 1991; U.S. Pat. No. 4,895,724, issued Jan. 23, 1990; and U.S. Pat. No. 4,675,189, issued Jun. 23, 1987, incorporated as if fully set forth herein by reference.

Sustained release oral dosage forms coated with bioadhesives can also be used. Examples are compositions disclosed in European Published Application EP 516141; U.S. Pat. No. 4,226,848; U.S. Pat. No. 4,713,243; U.S. Pat. No. 4,940,587; PCT International Application WO 85/02092; European Published Application 205282; Smart J D et al. (1984) J Pharm Pharmacol 36:295-9; Sala et al. (1989) Proceed Intern Symp Control Rel Bioact Mater 16:420-1; Hunter et al. (1983) International Journal of Pharmaceutics 17:59-64; "Bioadhesion--Possibilities and Future Trends, Kellaway," Course No. 470, May 22-24, 1989, incorporated as if fully set forth herein by reference.

Commercially available sustained release formulations and devices include those marketed by ALZA Corporation, Palo Alto, Calif., under tradename ALZET, INFUSET, IVOS, OROS, OSMET, or described in one or more U.S. Pat. No. 5,284,660, issued Feb. 9, 1994; U.S. Pat. No. 5,141,750, issued Aug. 25, 1992; U.S. Pat. No. 5,110,597, issued May 5, 1992; U.S. Pat. No. 4,917,895, issued Apr. 17, 1990; U.S. Pat. No. 4,837,027, issued Jun. 6, 1989; U.S. Pat. No. 3,993,073, issued Nov. 23, 1976; U.S. Pat. No. 3,948,262, issued Apr. 6, 1976; U.S. Pat. No. 3,944,064, issued Mar. 16, 1976; and U.S. Pat. No. 3,699,963; International Applications PCT/US93/10077 and PCT/US93/11660; and European Published Applications EP 259013 and EP 354742, incorporated as if fully set forth herein by reference.

Administration of the tolerizing antigen can also be affected by transforming cells of the mucosal tissue with a nucleic acid capable of encoding the antigen, and expression within cells of the mucosa. Methods for inducing mucosal immunity

using local expression of nucleic acid constructs are disclosed in U.S. Patent Application No. 10/076900 to Weiner et al, filed February 4, 2004, incorporated as if fully set forth herein by reference.

Sustained release compositions and devices are suitable for use in the present invention because they serve to prolong contact between the antigen and the gut-associated lymphoid tissue (GALT) and thus prolong contact between the antigen and the immune system. In addition, sustained release compositions obviate the need for discrete multi-dose administration of the antigen and permit the required amount of antigen to be delivered to GALT in one or two daily doses. This is anticipated to improve patient compliance.

Orally administrable pharmaceutical formulations containing one or more of a β_2GPI protein, a therapeutically effective β_2GPI fragment, and/or a therapeutically effective β_2GPI analog are prepared and administered to mammals who have manifested symptoms of vascular disorder, such as atherosclerosis. Additionally, subjects who are at risk for developing a vascular disorder, i.e., have a genetic predisposition to developing the disorder, as determined through suitable means, such as genetic studies and analysis, are treated with similar oral preparations.

Pharmaceutical formulations for oral or enteral administration to treat vascular disorders are prepared from a β_2GPI protein, a therapeutically effective β_2GPI fragment, and/or a therapeutically effective β_2GPI analog and a pharmaceutically acceptable carrier suitable for oral ingestion. The quantity of a β_2GPI protein, a therapeutically effective β_2GPI fragment, and/or a therapeutically effective β_2GPI analog in each daily dose may be between 0.001 mg and 1000 mg per day. However, the total dose required for treatment can vary according to the individual and the severity of the condition. This amount can be further refined by well-known methods such as establishing a matrix of dosages and frequencies of administration.

For by-inhalation administration (i.e., delivery to the bronchopulmonary mucosa) suitable sprays and aerosols can be used, for example using a nebulizer such as those described in U.S. Pat. No. 4,624,251 issued Nov. 25, 1986; U.S. Pat. No. 3,703,173 issued Nov. 21, 1972; U.S. Pat. No. 3,561,444 issued Feb. 9, 1971; and

U.S. Pat. No. 4,635,627 issued Jan. 13, 1971, incorporated as if fully set forth herein by reference. The aerosol material is inhaled by the subject to be treated.

Other systems of aerosol delivery, such as the pressurized metered dose inhaler (MDI) and the dry powder inhaler as disclosed in Newman S P in Aerosols and the Lung, S W Clarke S W and D Davis, eds. pp. 197-224, Butterworths, London, England, 1984, can be used when practicing the present invention.

Aerosol delivery systems of the type disclosed herein are available from numerous commercial sources including Fisons Corporation (Bedford, Mass.), Schering Corp. (Kenilworth, N.J.) and American Pharmoseal Co. (Valencia, Calif.).

Formulations for nasal administration can be administered as a dry powder or in an aqueous solution. Preferred aerosol pharmaceutical formulations may comprise for example, a physiologically acceptable buffered saline solution containing a β_2GPI protein, a therapeutically effective β_2GPI fragment, and/or a therapeutically effective β_2GPI analog of the present invention.

Dry aerosol in the form of finely divided solid comprising a β_2GPI protein, a therapeutically effective β_2GPIfragment, and/or a therapeutically effective β_2GPI analog in particle form, which particles are not dissolved or suspended in a liquid are useful in the practice of the present invention. The antigen may be in the form of dusting powders and comprise finely divided particles having an average particle size of between about 1 and 5 μm, preferably between 2 and 3 μm. Finely divided antigen particles may be prepared by pulverization and screen filtration using techniques well known in the art. The particles may be administered by inhaling a predetermined quantity of the finely divided material, which can be in the form of a powder.

Specific non-limiting examples of the carriers and/or diluents that are useful in the pharmaceutical formulations of the present invention include water and physiologically acceptable buffered saline solutions such as phosphate buffered saline solutions pH 7.0-8.0.

The mucosally administered formulation of the present invention may include a thermosetting gel which increases in viscosity at body temperature upon contact with the mucosa.

Formulations for buccal administration can include mucoadhesive mixed with effective amounts of a β_2GPI protein, a therapeutically effective β_2GPI fragment,

and/or a therapeutically effective β_2GPI analog. Effective amounts are anticipated to vary according to the formulation employed. For formulation administered by inhalation, the effective amount is likely to be less than that of the oral dose.

Preferably, the duration of treatment in humans should be a minimum of two weeks, and typically three months, and may be continued indefinitely or as long as benefits persist. The treatment may be discontinued if desired (in the judgment of the attending physician) and the patient monitored for signs of relapse. If clinical symptoms or other disorder indicators show that the patient is relapsing, treatment may resume.

As will be understood by those skilled in the art, the dosage will vary with the antigen administered and may vary with the sex, age, and physical condition of the patient as well as with other concurrent treatments being administered. Consequently, adjustment and refinement of the dosages used and the administration schedules will preferably be determined based on these factors and especially on the patient's response to the treatment. Such determinations, however, require no more than routine experimentation, as illustrated in Examples provided below.

Administration of a β_2GPI protein, a therapeutically effective β_2GPI fragment, and/or a therapeutically effective β_2GPI analog can be conjoined with mucosal administration of one or more enhancers, i.e. substances that enhance the tolerizing effect of the a β_2GPI protein, a therapeutically effective β_2GPI fragment, and/or a therapeutically effective β_2GPI analog antigen. Such enhancers include lipopolysaccharide (LPS), Lipid A (as described in U.S. application Ser. No. 08/202,677, published as WO 91/01333), IL-4, IL-10 and Type I interferon (See, e.g., U.S. application Ser. Nos. 08/420,980 and 08/420,979 and WO 95/27499 and WO 95/27500). Other suitable enhancers of mucosal tolerance are a group of β-1,3, β-1-6 glucan products described in detail in U.S. Patent Application Nos. 20030104010 and 20020009463 to Raa et al., and the oral tolerance inducing agents disclosed by Holmgren et al in US Patent No. 5,681,571, all incorporated as if fully set forth herein by reference) As used in the preceding sentence, "conjoined with" means before, substantially simultaneously with, or after administration of these antigens. Naturally, administration of the conjoined substance should not precede nor follow administration of the antigen by so long an interval of time that the relevant effects of

the substance administered first have worn off. Therefore, enhancers should usually be administered within about 24 hours before or after the β_2GPI protein, a therapeutically effective β_2GPI fragment, and/or a therapeutically effective β_2GPI analog antigen and preferably within about one hour.

As used herein the term "therapeutically effective fragment" refers to a peptide or polypeptide containing partial amino acid sequences or moieties of β_2GPI proteins possessing the ability to treat a vascular disorder. Preferably, such fragments are able to suppress or prevent an inflammatory response upon mucosal administration. Such fragments need not possess all the immunogenic properties of the entire β_2GPI protein. By way of non-limiting example, when MBP is administered parenterally to susceptible mice in the presence of an adjuvant, it induces experimental allergic encephalomyelitis RAE). It is known that certain non-disease-inducing fragments of MBP (i.e., fragments of MBP which do not induce EAE when administered parenterally with an adjuvant) nevertheless possess autoimmune-suppressive activity when administered orally (or enterally) or in aerosol form to mammals suffering from EAE. Examples of such fragments are reported in U.S. patent application Ser. No. 07/065,734, filed Jun. 24, 1987, and International Patent Application No. PCT/US88/02139, filed Jun. 24, 1988. Similarly, the present inventors have found that while immunization with a single subcutaneous administration of β_2GPI and adjuvant induced atherogenesis in LDLR-/- mice (George, et al, Circulation, 1998; 98:1108-1115), mucosal administration of the same β2GPI antigen resulted in reduced plaque formation and inhibition of development of atherosclerosis (see U.S. Patent Application 10/450,370 to Harats et al, filed Jan. 3, 2002, and PCT IL02/00005 to Harats et al., filed Jan. 3, 2002). Therapeutically effective fragments and analogs can be identified by observing a change in cytokine release profile, such as illustrated in the Examples or in other in vitro or in vivo assays which are predictive of a human vascular disorder and from which agents can be selected which alleviate detectable symptoms of the disorder. Cytokines can be measured using routine assays, including commercially available immunoassays such as radioimmunoassay (RIA), enzyme-linked immunosorbent assay (ELISA) and RT-PCR.

As used herein the term "therapeutically effective derivative" of β_2GPI is defined as β_2GPI protein peptides or their therapeutically effective fragments (e.g., inflammatory response-suppressive fragments) which possess the same biological activity, i.e., the ability to treat the condition, e.g., by eliminating or suppressing the inflammatory response, upon mucosal administration, either nasally, orally, or enterally. By way of non-limiting example, the term includes peptides having amino acid sequences which differ from the amino acid sequence of the β_2GPI peptide or therapeutically effective fragments thereof by one or more amino acid residues (while still retaining the inflammatory response-suppressive activity of the β_2GPI protein peptide or fragment) as well as compounds or compositions which mimic the inflammatory response-suppressive activity of the β_2GPI protein peptide in its ability to suppress or alleviate the symptoms of the disorder.

The tolerance induced by the autoimmune-suppressive agents of this invention is dose-dependent. Dose dependency was also seen in the autoimmune arthritis system. Moreover, the mucosal administration of an irrelevant antigen (i.e., one not implicated in an autoimmune disease, such as ovalbumin (OVA) peptide, histone protein, or certain synthetic fragments of MBP) has no effect on the clinical manifestation of the autoimmune disease.

Various animal models have been developed for the study of atherosclerosis and are predictive of human atherosclerosis. Among the more common models are those in which inbred strains of mice have been rendered deficient for either the LDL receptor (LDLR -/-) or apolipoprotein E (apo E -/-) by a gene knockout. The LDL receptor is a 160 kDa glycoprotein responsible for the transfer of LDL out of the plasma and into the cytoplasm of virtually all cell types. The major site of LDL uptake and catabolism is the liver. LDLR-/- mice created on a C57BL/6 background develop accelerated atherosclerosis when fed a high cholesterol diet, but not when fed a regular chow diet. By contrast, wild-type C57BL/6 mice typically do not develop accelerated atherosclerosis on either a high cholesterol or a regular chow diet.

In one recent study, the present inventors found that LDLR-/- C57BL/6 mice immunized subcutaneously with 10 or 100 μg of heat-killed Mycobacterium tuberculosis and maintained on a normal chow diet for three months developed

significantly larger fatty streaks than negative control mice immunized with bovine serum albumin (Afek A et al. J Autoimmun 2000; 14:115-121). These and other animal models can be used to select β_2GPI peptides that are useful in accordance with the methods of the invention.

A therapeutically effective amount means an amount of active ingredients effective to induce an immune response thus preventing, alleviating or ameliorating symptoms of a disorder (e.g., atherosclerosis).

Ascertaining the optimum regimen for administering the active ingredient(s) is determined in light of the information disclosed herein and well known information concerning administration of mucosally active antigens, and autoantigens. Routine variation of dosages, combinations, and duration of treatment is performed under circumstances wherein the severity of atheromatous development can be measured. Useful dosage and administration parameters are those that result in reduction in inflammatory reaction, including a decrease in number of autoreactive T-cells, or in the occurrence or severity of at least one clinical or histological symptom of the disease.

In further preferred embodiments of the present invention, cytokine and non-cytokine synergists can be conjoined in the treatment to enhance the effectiveness of mucosal tolerization with plaque associated molecules. Oral and parenteral use of other cytokine synergists (Type I interferons) has been described in PCT/US95/04120, filed Apr. 07, 1995. Administration of Th2 enhancing cytokines is described in PCT application no. PCT/US95/04512, filed Apr. 07, 1995. For example, IL-4 and IL-10 can be administered in the manner described in PCT/US95/04512.

Toxicity and therapeutic efficacy of the active ingredients described herein can be determined by standard pharmaceutical procedures *in vitro*, in cell cultures or experimental animals. The data obtained from these *in vitro* and cell culture assays and animal studies can be used in formulating a range of dosage for use in human. The dosage may vary depending upon the dosage form employed and the route of administration utilized. The exact formulation, route of administration and dosage can be chosen by the individual physician in view of the patient's condition. (See e.g., Fingl, *et al.*, 1975, in "The Pharmacological Basis of Therapeutics", Ch. 1 p.1).

Dosage amount and interval may be adjusted individually to provide mucosal levels of the active ingredient that are sufficient to induce tolerance. The "tolerizing dosage" will vary for each preparation, but can be estimated from *in vitro* data. Dosages necessary to achieve tolerizing dosage will depend on individual characteristics and route of administration. Detection assays can be used to determine plasma concentrations.

Depending on the severity and responsiveness of the condition to be treated, dosing can be of a single or a plurality of administrations, with course of treatment lasting from several days to several weeks or until cure is effected or diminution of the disease state is achieved.

The amount of a composition to be administered will, of course, be dependent on the subject being treated, the severity of the affliction, the manner of administration, the judgment of the prescribing physician, etc.

Compositions of the present invention may, if desired, be presented in a pack or dispenser device, such as an FDA approved kit, which may contain one or more unit dosage forms containing the active ingredient. The pack may, for example, comprise an inhaler. The pack or inhaler may be accompanied by instructions for administration. The pack or inhaler may also be accommodated by a notice associated with the container in a form prescribed by a governmental agency regulating the manufacture, use or sale of pharmaceuticals, which notice is reflective of approval by the agency of the form of the compositions or human or veterinary administration. Such notice, for example, may be of labeling approved by the U.S. Food and Drug Administration for prescription drugs or of an approved product insert. Compositions comprising a preparation of the invention formulated in a compatible pharmaceutical carrier may also be prepared, placed in an appropriate container, and labeled for treatment of an indicated condition, as is further detailed above.

Thus, according to another aspect of the present invention there is provided an article of manufacture, packaged and identified for use in modulating an immune response to an atheroma plaque antigen in a subject in need thereof. The article of manufacture includes a packaging material and a mucosal tolerance-inducing amount of an active ingredient selected from the group consisting of beta$_2$-glycoprotein-1

(β_2GPI) or a derivative of β_2GPI, the packaging material including a label or package insert indicating that the mucosal tolerance-inducing amount of the active ingredient is for modulating an immune response to an atheroma plaque antigen in the subject via mucosal administration.

According to yet a further aspect of the present invention there is provided an article of manufacture, packaged and identified for use in the prevention and/or treatment of a vascular condition in a subject in need thereof. The article of manufacture includes a packaging material and a mucosal tolerance-inducing amount of an active ingredient selected from the group consisting of beta$_2$-glycoprotein-1 (β_2GPI) or a derivative of β_2GPI, the packaging material including a label or package insert indicating that the mucosal tolerance-inducing amount of the active ingredient is for prevention and/or treatment of a vascular condition in the subject via mucosal administration.

Additional objects, advantages, and novel features of the present invention will become apparent to one ordinarily skilled in the art upon examination of the following examples, which are not intended to be limiting. Additionally, each of the various embodiments and aspects of the present invention as delineated hereinabove and as claimed in the claims section below finds experimental support in the following examples.

EXAMPLES

Reference is now made to the following examples, which together with the above descriptions, illustrate the invention in a non limiting fashion.

Generally, the nomenclature used herein and the laboratory procedures utilized in the present invention include biochemical and immunological techniques. Such techniques are thoroughly explained in the literature. See, for example, "Cell Biology: A Laboratory Handbook", Volumes I-III Cellis, J. E., ed. (1994); "Current Protocols in Immunology" Volumes I-III Coligan J. E., ed. (1994); Stites et al. (eds), "Basic and Clinical Immunology" (8th Edition), Appleton & Lange, Norwalk, CT (1994); Mishell and Shiigi (eds), "Selected Methods in Cellular Immunology", W. H. Freeman and Co., New York (1980); available immunoassays are extensively described in the patent and scientific literature, see, for example, U.S. Pat. Nos.

3,791,932; 3,839,153; 3,850,752; 3,850,578; 3,853,987; 3,867,517; 3,879,262; 3,901,654; 3,935,074; 3,984,533; 3,996,345; 4,034,074; 4,098,876; 4,879,219; 5,011,771 and 5,281,521; and "Methods in Enzymology" Vol. 1-317, Academic Press; Marshak et al., all of which are incorporated by reference as if fully set forth herein. Other general references are provided throughout this document. The procedures therein are believed to be well known in the art and are provided for the convenience of the reader. All the information contained therein is incorporated herein by reference.

General Materials and Methods

Animals: Apo-E deficient mice used in these experiments are from the atherosclerosis prone strain C57BL/6J-Apoe^{tm1unc}. Mice homozygous for the Apoe^{tm1unc} mutations show a marked increase in total plasma cholesterol levels which is unaffected by age or sex. Fatty streaks in the proximal aorta are found at 3 months of age. The lesions increase with age and progress to lesions with less lipid but more elongated cells, typical of a more advanced stage of pre-atherosclerotic lesion.

LDL-RD mice (hybrids of a cross between the C57BL/6J and 129Sv strains) were previously created with homologous recombination as described by Ishibashi et al. (J Clin Invest 1993;92:883-93). The LDL-RD mice have less highly elevated plasma cholesterol levels than the Apo-E deficient mice, but are also susceptible to atherosclerosis. All mice that were used in the experiment were females of age 6 weeks. The LDL-RD mice were obtained from Jackson Laboratories and bred at the local animal facility, as described for the Apo-E deficient mice.

Strain Development: The Apoe^{tm1unc} mutant strain was developed in the laboratory of Dr. Nobuyo Maeda at University of North Carolina at Chapel Hill. The 129-derived E14Tg2a ES cell line was used. The plasmid used is designated as pNMC109 and the founder line is T-89. The C57BL/6J strain was produced by backcrossing the Apoe^{tm1unc} mutation 10 times to C57BL/6J mice (11,12). The mice were maintained at the Sheba Hospital Animal Facility (Tel-Hashomer, Israel) on a 12-hour light/dark cycle, at 22-24°C and fed a normal fat diet of laboratory chow (Purina Rodent Laboratory Chow No. 5001) containing 0.027% cholesterol, approximately 4.5% total fat, and water, ad libitum. "Western diet" (TD 96125,

Harlan Teklad, 42% calories from fat, 43% from carbohydrates and 15% from protein) describes a standardized, high fat atherogenic diet.

Nasal Tolerance: Nasal tolerance was induced by intranasal administration of oxidized LDL, β_2GPI or HSP65, in a total volume of 10 µl PBS. Intranasal administration was performed on mildly sedated mice (12-16 weeks old), each mouse receiving 3 doses of antigen per dose, in the indicated concentrations, every other day. Atherogenesis was induced by 5 weeks of a Western diet, initiated on the day following the last intranasal administration. Controls received equal amounts of BSA and/or PBS, as indicated, in an identical regimen. Sera were obtained for assessment of cholesterol and triglyceride levels from all mice, and the mice were sacrificed for evaluation of atherosclerosis, as described hereinbelow, after 5 weeks Western diet.

Oral Tolerance: For comparison, oral tolerance to plaque-associated molecules was induced by feeding 3 doses of antigen every other day (for a detailed account of induction of oral tolerance, see US Pat Application No 09/806,400 to Shoenfeld et al filed Sept 30, 1999), in a similar regimen to the nasal tolerance. LDL-RD mice were fed by a nasogastric tube, five doses (every other day) of human or bovine β_2GPI in PBS in two different concentrations (500 and 50 µg/dose). Control mice were either fed an irrelevant antigen (BSA; 50 µg) or not fed. One day following the last feeding, all mice were switched from chow-diet to Western diet and sacrificed 4 weeks later.

Antigen Preparation

β_2GPI: Human and bovine β2GPI was purified from the serum of a healthy adult as described (Gharavi, et al, J Clin Invest 1992;92:1105-09; George et al Circulation 1998;15:1108-15). To ensure purity, pooled plasma was subsequently chromatographed on a heparin-SEPHAROSE column, on a DEAE-cellulose column, and on an anti- β_2GPI affinity column. To remove any contamination by IgGs, the β2GPI-rich fraction was further passed through a protein A-SEPHAROSE column.

Oxidized LDL: Human LDL (density=1.019- 1.063g/l) was prepared from sera of fasting individuals by preparative ultracentrifugation (50,000 rpm/min, 22 min), washing, dialysis against 150mM EDTA, pH 7.4, filtration (0.22 µm pore size) to remove aggregation, and storage under nitrogen. LDL oxidation was performed by

incubation of dialyzed, EDTA free LDL with copper sulfate (10 μM) for 24 hours at 37° C. Lipoprotein oxidation was confirmed by analysis of thiobarbituric acid-reactive substances (TBARS) which measures malondialdehyde (MDA) equivalents.

HSP65: Recombinant mycobacterial HSP-65, prepared as described (Prohaszka Z et al, Int Immunol 1999;11:1363-70) was kindly provided by Dr. M. Singh, Braunschweig, Germany.

Immunization: Subcutaneous immunization with human β_2GPI: Human β_2GPI was prepared from human plasma pool as described above. For immunization, human β_2GPI was dissolved in PBS and mixed with equal volumes of Freund's incomplete adjuvant. Immunizations were performed by single subcutaneous injection of 10μg antigen/mouse in 0.1ml volume. Three days following the last mucosal administration of plaque associated molecules the mice received one immunization, and were sacrificed 10 days post immunization.

Cholesterol Level Determination: At the completion of the experiment, 1-1.5 ml of blood was obtained by cardiac puncture, 1000U/ml heparin was added to each sample and total plasma cholesterol levels were determined using an automated enzymatic technique (Kit No. 816302, Boehringer, Mannheim, Germany).

FPLC Analysis: Fast Protein Liquid Chromatography analysis of cholesterol and lipid content of lipoproteins was performed using Superose 6 HR 10/30 column (Amersham Pharmacia Biotech, Inc, Peapack, NJ) on a FPLC system (Pharmacia LKB. FRAC-200, Pharmacia, Peapack, NJ). A minimum sample volume of 300 μl (blood pooled from 3 mice was diluted 1:2 and filtered before loading) was required in the sampling vial for the automatic sampler to completely fill the 200 μl sample loop. Fractions 10-40 were collected, each fraction contained 0.5 ml. A 250 μl sample from each fraction was mixed with freshly prepared cholesterol reagent or triglyceride reagent respectively, incubated for 5 minutes at 37°C and assayed spectrophotometrically at 500nm.

Assessment of Atherosclerosis: Quantification of atherosclerotic fatty streak lesions was done by calculating the lesion size in the aortic sinus as previously described and by calculating the lesion size in the aorta. Briefly, after perfusion with saline Tris EDTA, the heart and the aorta were removed from the animals and the peripheral fat cleaned carefully. The upper section of the heart was embedded in

OCT medium (10.24% w/w polyvinyl alcohol; 4.26% w/w polyethylene glycol; 85.50% w/w nonreactive ingredients) and frozen. Every other section (10 μm thick) throughout the aortic sinus (400 μm) was taken for analysis. The distal portion of the aortic sinus was recognized by the three valve cusps that are the junctions of the aorta to the heart. Sections were evaluated for fatty streak lesions after processing and staining with oil-red O, according to Paigen et al (Atherosclerosis, 1987;68:231-40). Lesion areas per section were scored on a grid by an observer counting unidentified, numbered specimens. The aorta was dissected from the heart and surrounding adventitious tissue was removed. Fixation of the aorta and Sudan staining of the vessels were performed as previously described.

Immunohistochemistry of atherosclerotic lesions: Immunohistochemical staining for CD3, macrophages and β_2GPI content were done on aortic sinus 5-μm-thick frozen sections. Primary antibodies used for probing were rat anti-mouse CD3, rat anti-mouse Mac-1 and a polyclonal rat anti-mouse β_2GPI antibodies (George et al Circulation 2000;102:1822-7). Slides were developed with the three amino-9-ethylcarbonasole (AEC) substrate. Sections were counterstained with hematoxylin. Spleen sections were used as a positive control. Staining in the absence of 1st or 2nd antibody was used as a negative control. β_2GPI presence were evaluated by its occupancy of plaque area by computerized morphometry as described previously for VCAM-1 (George et al, Circ Res. 2000;86:1203-10).

Proliferation assays: Mice were exposed to the tested antigen as described for assessment of atherosclerosis, and then immunized (one to three days following the last exposure) subcutaneously with 10 μg β_2GPI in 0.1 ml PBS, prepared from purified human β_2GPI as described above.

Proliferation was assayed ten days after immunization with the β_2GPI as follows: Draining inguinal lymph nodes were prepared by mashing the tissues on 100 mesh screens. Red blood cells were lysed with cold sterile double distilled water (6ml) for 30 seconds and 2ml of NaCl 3.5% was added. Incomplete medium was added (10ml), cells were centrifuged for 7 min at 1,700 rpm, resuspended in RPMI medium and counted in a haemocytometer at 1:20 dilution (10μl cells + 190μl Trypan Blue). Proliferation was measured by the incorporation of [^{3}H] Thymidine into DNA in triplicate samples of 100μl of the packed cells ($1x10^6$ cells/ml) in a 96

well microtiter plate. Triplicate samples of β_2GPI (10μg/ml, 100μl/well) or BSA were added, cells incubated for 72 hours (37°C, 5% CO_2 and ~98% humidity) and 10μl ^{3}[H] Thymidine (0.5μCi/well) was added. After an additional 12-24 hours of incubation the cells were harvested and transferred to glass fiber filters using a cell harvester (Brandel) and counted using β-counter (Lumitron). Proliferation was measured by the incorporation of [^{3}H] thymidine into DNA during the final 12 h of incubation. The results are expressed as the stimulation index (S.I.): the ratio of the mean radioactivity (cpm) of the antigen to the mean background (cpm) obtained in the absence of the antigen. Standard deviation was always <10% of the mean cpm.

For assessment of mucosal tolerance with β_2GPI on reactivity to oxidized LDL, mice (n=4) were exposed to tolerizing doses of β_2GPI or BSA in five doses, as described hereinabove. One day following the last dose, all mice were immunized with human ox-LDL or BSA (10μg/ml) and draining lymph nodes collected 10 days later. Proliferation in response to oxLDL stimulus was assessed as above, following 72 hours incubation with oxLDL.

IFN-γ, IL-4, IL-10 and TGF-β secretion in tolerized lymph nodes: Conditioned medium was obtained from the lymph node proliferation experiments following 48 h of culture in the presence of β_2GPI. IFN-g, IL-4, IL-10 and TGF-h concentrations were determined by ELISA kits according to the manufacturer's suggestions (R&D Systems Inc., Minneapolis, MN).

RT-PCR analysis of cytokine expression: 7-9 week old male ApoE-KO mice were tolerized by oral administration of β_2GPI in 5 feedings, every other day, of human β_2GPI (100 μg/mouse) or PBS, as control, by gavage, as detailed hereinabove. Three days following the oral administration of β_2GPI, the mice were sacrificed, aortas collected and processed for RT-PCR analysis of the expression of anti-inflammatory Th2 type cytokine IL-10 and the proinflammatory Th1-type cytokine IFN-γ. The RT-PCR analysis was performed according to the protocol as described in detail by Colle et al (Journal of Immunol Methods 1997;175-184). Briefly, RNA was extracted from the aortal tissue and reverse transcribed according to well-known, standard protocols, and the transcription products subjected to PCR amplification using the following primers: IL-10- forward primer 5' CTGGACAACATACTGCTAACCGAC 3' (SEQ ID NO: 1), located at nucleotide

positions 256-278 of IL-10 (GenBank Accession No. NM_010548 ; SEQ ID NO: 2) and reverse primer 5'ATTCATTCAYGGCCTTGTAGACACC 3' (SEQ ID NO: 3), located at nucleotide positions 532-556 of IL-10 (SEQ ID NO: 2); IFN-γ- forward primer 5' CTTCTTCAGCAACAGCAAGGCGAAAA 3' (SEQ ID NO: 4), located at nucleotide positions 372-397 of IFN-γ (GenBank Accession No. NM_008337 ; SEQ ID NO: 5), reverse primer 5' CCCCCAGATACAACCCCGCAATCA 3' (SEQ ID NO: 6), located at nucleotide positions 804-827 of IFN-γ (SEQ ID NO: 5); and β-actin- forward primer 5' GGACTCCTATGTGGGTGACGAGG 3' (SEQ ID NO: 7), located at nucleotide positions 230-252 of β-actin (GenBank Accession No. NM 007393; SEQ ID NO: 8), and reverse primer 5' GGGAGAGCATAGCCCTCGTAGAT 3' (SEQ ID NO: 9), located at nucleotide positions 573-579 of β-actin (SEQ ID NO: 8). The resultant amplified IL-10, IFN-γ and β-actin transcripts were separated by electrophoresis on an agarose gel, and visualized by ethidium bromide staining.

Detection of anti-β_2GPI antibodies and antibody isotypes: β_2GPI (10 μg/ml) was coated onto flat bottom 96-well ELISA plates (Nalge-Nunc, Int. Rochester, NY) by overnight incubation and the assay was performed as previously described (George et al, Circulation, 1998; 15:1108-15) IgG, IgA and IgM isotypes in the sera of β_2GPI tolerant and non-tolerant mice were determined by an ELISA kit (Southern Biotechnology Associates, Birmingham, AL, USA) according to the manufacture's instructions.

Statistical Analysis: A one-way ANOVA test was used to compare independent values. $p<0.05$ was accepted as statistically significant.

EXAMPLE 1

Inhibition of atherogenesis in genetically predisposed (LDL receptor-deficient) mice by induction of nasal tolerance with low doses of the plaque associated molecules oxidized LDL, human β_2GPI and HSP 65

The present inventors here demonstrate that mucosal administration, via nasal exposure, to low doses of the plaque associated molecules oxidized LDL, β_2GPI and HSP 65 provides induction of immune tolerance to the antigens, and significant inhibition of atherogenesis. Thus, nasal exposure to purified, oxidized human LDL,

human β_2GPI and recombinant mycobacterial HSP 65 were compared for their effectiveness in suppressing atherogenesis in LDL-RD mice. 63 male 9-13 week old LDL RD mice were divided into 5 groups. In group A (HSP-65)(n=12) nasal tolerance was induced as described in Materials and Methods by administration of recombinant mycobacterial HSP 65 suspended in PBS (10 μg/mouse/10μl) for 5 days every other day. In group B (H-oxLDL)(n=14) nasal tolerance was induced as described in Materials and Methods by administration of 10 μg/mouse/10μl oxidized purified human LDL, suspended in PBS, every other day for 5 days. Mice in group C (β_2GPI)(n=13) received 10 μg/mouse/10μl human β_2GPI per mouse, administered intranasally as described in Materials and Methods, every other day for 5 days. Mice in group D (BSA)(n=12) received 10 μg/mouse/10μl bovine serum albumin (BSA) per mouse, administered intranasally as described in Materials and Methods, every other day for 5 days. Mice in group E (PBS)(n=12) received 10μl PBS per mouse, administered intranasally. Mice were bled prior to feeding (Time 0) and at the conclusion of the experiment (End) for determination of lipid profile. Atherosclerosis was assessed in heart and aorta as described above, 8 weeks after the last feeding. Mice were weighed every 2 weeks during the experiment. All mice were fed water ad libitum and a normal chow-diet containing 4.5% fat by weight (0.02% cholesterol), up to the final antigen exposure, and then a "Western" diet until sacrifice.

Table 1: Inhibition of atherogenesis in LDL receptor-deficient mice by intranasal administration of exceedingly low doses of plaque associated molecules

Time		HSP-65	H-oxLDL	Hβ_2GPI	BSA	PBS	
Day 0	Weight (gr) (Mean±SE)	22.6 ±0.8	22.3 ±0.5	22.3 ±0.7	21.8 ±0.7	21.7 ±0.5	p=0.833
	Chol (mg/dL) (Mean±SE)	237 ±13	230 ±10	230 ±14	236±19	227±14	P=0.986
	TG(mg/dL) (Mean±SE)	150 ±19	178 ±17	162 ±18	185±22	160±15	P=0.664
END	Weight (gr) (Mean±SE)	26.8±0.9	28.2±1.0	29.2±1.5	25.5±1.0	26.3±1.3	P=0.157
	Chol (mg/dL) (Mean±SE)	1181 ±114	1611 ±119	1601 ±125	1470±183	1606 ±181	P=0.197
	TG(mg/dL) (Median)	288	275	380	315	403	P=0.416
	Sinus Lesion	44375	43393	46250	120500	128182	P<0.001

	(μm^2) (Mean±SE)	±5437	±4107	±4486	±8746	±9102	

Note: "Weight" is weight in grams; "Chol" is serum cholesterol and "TG" is serum triglycerides, expressed in mg/dL.

As can be seen from Figure 1, the results depicted in Table 1 demonstrate the strikingly effective inhibition of atherogenesis measured in the tissues of mice receiving mucosal (nasal) exposure to low doses (10 μg/ mouse) of the plaque associated molecules, compared to control mice exposed to sham antigen (BSA) or PBS. Furthermore, nasal tolerance is specific in its mode of protection: clearly, induction of nasal tolerance has no significant effect on other general parameters measured, such as weight gain, triglyceride or cholesterol blood levels. Thus, the antigenic plaque associated molecules oxidized LDL, β_2GPI and HSP 65 are highly potent inducers of mucosal tolerance, when administered nasally, with surprisingly low doses (10 μg/ mouse) and brief exposure (3 days) of significant (greater than 65%) and consistent protection from atherogenesis in these genetically susceptible LDL receptor-deficient mice.

EXAMPLE 2

Superior inhibition of atherogenesis in genetically predisposed (LDL-RD) mice by induction of nasal tolerance with HSP 65

The present inventors here demonstrate, that mucosal administration, by nasal exposure to exceedingly low doses of the plaque associated molecule HSP 65 provides superior induction of tolerance to the antigen, and inhibition of atherogenesis. Thus, nasal exposure to a low dose and an exceedingly low dose of recombinant human HSP 65 were compared for their effectiveness in suppressing atherogenesis in LDL-RD mice. 58 male 12-16 week old LDL-RD mice were divided into 4 groups. In group A (HSP-65 high)(n=14) nasal tolerance was induced as described in Materials and Methods by intranasal administration of 10μg/mouse/10μl recombinant human HSP 65 suspended in PBS for 5 days every other day. In group B (HSP-65 low)(n=16) nasal tolerance was induced as described in Materials and Methods by administration of 1 μg/mouse/10μl recombinant human HSP 65 suspended in PBS every other day for 5 days. Mice in group C (BSA)(n=14) received 1 μg/mouse/10μl BSA per mouse, administered intranasally, every other day

for 5 days. Mice in group D (PBS)(n=14) received 10μl PBS per mouse, administered intranasally. Mice were bled prior to feeding (Time 0) and at the conclusion of the experiment (End) for determination of lipid profile. Atherosclerosis was assessed in heart and aorta as described above, 8 weeks after the last feeding. Mice were weighed every 2 weeks during the experiment. All mice were fed water ad libitum and a normal chow-diet containing 4.5% fat by weight (0.02% cholesterol), up to the final antigen exposure, and then a "Western" diet until sacrifice.

Table 2: Superior inhibition of atherogenesis in LDL-receptor-deficient mice by intranasal administration of human HSP 65

		HSP65 10 μg/Mouse N=12	*HSP65 1 μg/Mouse N=16*	*BSA 100 μg/Mouse N=11*	**PBS** *N=10*	**Statistics**
End	Wt	28.4 ±1.0	26.9 ±0.9	27.7 ±0.5	28.7 ±0.7	P=0.363
	Chol	1073 ±65	1010 ±64	1009 ±74	1015 ±85	P=0.897
	Tg	348 ±32	315 ±46	316 ±32	390 ±44	P=0.564
	Sinus Les. μm^2	**22292 ±2691**	**17109 ±2053**	**54432 ±8201**	**47750 ±5779**	**P<0.05 Between HSP-65 and PBS or BSA**

Note: "Weight" is weight in grams; "Chol" is serum cholesterol and "TG" is serum triglycerides, expressed in mg/dL.

As can be seen from Figure 2, the results depicted in Table 2 demonstrate the superior effectiveness of inhibition of atherogenesis measured in the tissues of mice receiving nasal exposure to exceedingly low doses (1 μg/ mouse) of HSP 65, compared to control mice exposed to sham antigen (BSA) or PBS. Furthermore, nasal tolerance is specific in its mode of protection: clearly, induction of nasal tolerance, has no significant effect on other general parameters measured, such as weight gain, triglyceride or cholesterol blood levels. Thus, the antigenic plaque associated molecule HSP 65 is an extremely potent inducer of nasal tolerance, with even exceedingly low doses conferring significant (approximately 70%) protection from atherogenesis in genetically susceptible LDL-RD mice, greatly superior to the

protection achieved by induction of oral tolerance (30%, see US Patent Application No 09/806,400 to Shoenfeld et al filed Sept 30, 1999, the contents of which are incorporated by reference as if fully set forth herein).

EXAMPLE 3

Superior suppression of specific anti-β2GPI immune reactivity in genetically predisposed (LDL-RD) mice by mucosal administration of human β_2GPI

Tolerance induced by mucosal exposure to plaque-associated molecules may be mediated by suppression of specific immune responses to antigenic portions (epitopes) of these plaque associated molecules. Lymphocyte proliferation in response to mucosal (nasal and oral) exposure to human β_2GPI was measured in apoE-deficient mice. 9 male, 5 week old LDL-RD mice were divided into 3 groups. In group A (n=3) oral tolerance was induced with 100 μg/mouse β_2GPI suspended in 0.1 ml PBS, administered by gavage, as described above, every other day for 5 days. In group B (n=3) nasal tolerance was induced with 10 μg/mouse β_2GPI suspended in 10 μl PBS, administered intranasally as described above, every other day for 5 days. The mice in group C (n=3) received oral administration of 200 μl PBS every other day for 5 days. Immune reactivity was stimulated in all mice by immunization with human β_2GPI as described above in the Materials and Methods section, one day after the last feeding. Ten days after the immunization lymph nodes were collected for assay of proliferation (as expressed in the Stimulation Index SI). All mice were fed normal chow-diet containing 4.5% fat by weight (0.02% cholesterol) and water ad libitum.

Table 3: Intranasal pretreatment with purified human β2 GPI suppresses immune response to Human β2GPI in LDL receptor-deficient mice

	PBS	**H-β_2GPI (Oral)**	**H-β_2GPI (Nasal)**
S.I (Stimulation Index)	7.0±0.2	4.4±0.5	2.1±0.5

As can be seen from Figure 3, the results depicted in Table 3 demonstrate significant suppression of immune reactivity to human β_2GPI antigen, measured by inhibition of proliferation in the lymph nodes of LDL RD mice. Lymphocytes from mice receiving intranasal exposure to low atherogenesis-inhibiting doses (10 μg/ mouse) of human β_2GPI showed an exceedingly reduced stimulation index following immunization with β_2GPI, as compared to orally exposed and control (PBS) mice.

Since previous studies with induction of nasal tolerance have shown no significant effect on other parameters measured, such as weight gain, triglyceride or cholesterol blood levels, or immune competence (see abovementioned Examples), these results indicate a specific suppression of anti-β_2GPI immune reactivity. Thus, mucosal administration, by intranasal exposure, of the purified plaque associated molecule β_2GPI is a superior method of attenuating the cellular immune response to immunogenic and atherogenic plaque associated molecules in these genetically susceptible apoE-deficient mice.

EXAMPLE 4

Mucosal administration of β_2GPI effectively suppresses atherogenesis in LDL-Receptor deficient mice

LDL-receptor deficient (LDL-RD; LDLR -/-) mice created on a C57BL/6 background develop accelerated atherosclerosis when fed a high cholesterol diet, but not when fed a regular chow diet. In order to determine whether mucosal administration of the plaque antigen β_2GPI could suppress atherogenic processes, LDL-RD mice were fed low, oral tolerance inducing doses of human and bovine β_2GPI, and the assessed for alterations in response to diet.

Oral administration of Bovine or Human β2GPI is specifically antiatherogenic in LDL-RD mice: Oral administration (via gavage, as described hereinabove) of human β_2GPI at 50 μg and at 500 μg/dose were similarly effective in suppressing atherosclerosis in the LDL-RD mice (45% and 44% reduction, respectively, as compared with BSA-fed controls). Oral administration of bovine β_2GPI was also effective in reducing early atherosclerotic lesion size in both the low 500 μg and the exceedingly low 50 μg dosages (43% and 57% suppression, respectively (see Figs. 4 and 5). Oral administration of BSA did not alter lesion progression in comparison with PBS (Figs. 4 and 5).

In order to rule out non-specific, systemic effects of oral administration of β2GPI, the lipid profiles of the treated and control mice were determined.

Oral administration of Bovine or Human β_2GPI does not significantly influence total cholesterol or triglyceride levels: Table 4 shows the results of oral administration of 500 μg or 50 μg of bovine (B-β2) or human (H-β2) β_2GPI, or BSA,

to LDL-RD mice, as described above. No significant influence of β_2GPI administration, at either dose, on total cholesterol or triglycerides levels was observed, indicating that the antiatherogenic effects of oral β_2GPI administration do not result from alteration of the availability of plaque components.

Table 4: Lipid profile in weight in mice tolerized orally with β2GPI

		H-β2 GPI(500 μg)	H-β2 GPI (50 μg)	B-β2 GPI (500 μg)	B-β2 GPI (50 μg)	BSA (500 μg)	PBS
Start	Weight (g)	27.1±0.7	26.4±0.7	25.7±0.7	26.4±0.7	26.8±0.7	26.5±0.6
	Chol (mg/dl)	231±16	227±14	233±15	231±14	231±17	232±12
	Tg (mg/dl)	182±18	212±26	178±23	179±20	188±26	189±23
End	Weight (g)	31.4±1.0	32.0±0.6	29.8±0.9	30.8±0.8	30.5±0.8	31.6±0.6
	Chol (mg/dl)	1232±92	1237±71	1196±75	1200±76	1166±91	1250±90
	Tg (mg/dl)	415±62	342±51	318±55	313±51	293±45	424±54
	Aortic Sinus lesion (μm^2)	**27031±3387**	**26563±2370**	**20781±2290**	**27422±3007**	**48167±3340**	**50667±570**

H-β2—human β2GPI, B-β2—bovine β2GPI.

In order to assess whether oral administration of β2GPI affected endogenous β_2GPI, the content of plaque-expressed β2GPI was measured in mice receiving oral β_2GPI or BSA administration. Oral administration of β_2GPI (500 μg/dose) did not have a significant influence on the content of plaque-expressed β_2GPI (mean percent occupancy of 15±5) in comparison with BSA feeding (occupancy of 19±7%)(data not shown). In order to assess whether oral administration of β_2GPI affected the inflammatory phenotype of the fatty streaks, CD3-positiveand macrophage positive cells were measured in mice receiving oral β_2GPI or BSA administration. No effect of oral β_2GPI on the number of CD3 positive cells (0–5cells/plaque in all groups;

data not shown) or macrophage (Mac-1 positive) content of the fatty streaks was observed.

Thus, suppression of atherosclerosis by oral administration of β_2GPI was not the result of reduced availability of the autoantigen at the site of the lesion, nor of a change in the inflammatory immune-cell profile of the plaque lesions.

EXAMPLE 5

Superior inhibition of atherogenesis in genetically predisposed (apoE-deficient) mice by induction of mucosal tolerance with Mucosal Administration of β_2GPI

Mucosal administration of Human β_2GPI specifically inhibits progression of advanced atherogenic processes in ApoE-KO mice: Adult ApoE-KO mice develop advanced atherosclerotic lesions when fed an atherogenic "Western diet". In order to determine the protective effect of mucosal administration of β_2GPI on development of atherosclerosis, adult ApoE-KO mice were treated with oral administration of human β_2GPI. 20 week-old male ApoE-KO mice, having advanced atherosclerotic lesions were treated monthly with human β_2GPI (50μg/dose) or PBS (0.2 ml) in 5 oral administrations (by gavage, as described hereinabove) given every other day, for four months. Lesion area calculated from cryosections of the aortic sinus were compared between control untreated 20 week old mice, and mice following 16 weeks oral administration of β_2GPI or PBS.

As shown in Fig. 6, 16 weeks after initiating treatment, atherosclerosis (aortic lesions) in the PBS treated controls had progressed 124% over initial lesions in the 20 week old mice. Oral administration of 50μg human β_2GPI during the following 16 weeks inhibited the progression of atherosclerotic lesions (35% reduction) as compared to PBS control.

In order to rule out non-specific, systemic effects of oral administration of β_2GPI, the lipid profiles of the treated and control mice were determined.

Oral administration of Human β_2GPI does not significantly influence metabolic profile of mice having advanced atherosclerosis: Table 5 shows the results of oral administration of 50 μg of human (H-β2) β_2GPI, or PBS, to adult male Apo-E KO mice, as described above. No significant influence of β_2GPI

administration, at either dose, on body weight, total cholesterol or triglycerides levels was observed, indicating that the inhibition of atherosclerotic progression in adult male Apo-E KO by oral β_2GPI administration does not result from alteration of the availability of plaque components or lipid metabolism.

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Table 5: Lipid profile and weight in mice tolerized orally with β2GPI

		UNTREATED (week 16)	PBS	B-β2 GPI (50 µg/mouse)
Start t= 0	Weight (g)	25.5±0.7	265.5±0.7	25.5±0.4
	Chol (mg/dl)	361±28	361±20	360±17
	Tg (mg/dl)	76±9	76±5	71±17
End t= 16 weeks	Weight (g)	NA	28.1±0.6	29.6±0.8
	Chol (mg/dl)	NA	1494±21	505±42
	Tg (mg/dl)	NA	114±7	109±9
	Aortic Sinus lesion (μm^2)	**152321±6106**	**345000±18370**	**221042±14472**

H-β2—human β_2GPI.

EXAMPLE 6

10 ***Mucosal administration of β_2GPI specifically suppresses the immune response to β_2GPI and other plaque-related autoantigens in LDL-Receptor deficient mice***

Nicoletti et al (Mol Med. 2000;6, 283-90) have shown that tolerance to the antigens in oxidized LDL, brought about by neonatal administration of β_2GPI, led to clonal anergy/deletion of the oxLDL reactive cells and to consequent suppression of 15 atherosclerosis. The effect of oral administration of β_2GPI on the character of the immune response to β_2GPI and other plaque related autoantigens was assessed in LDL-RD mice.

Oral administration of β2GPI inhibits the cellular immune response to plaque related antigens: In order to assess the role of specific induction of immune 20 tolerance in the antiatherogenic effects of oral administration of β_2GPI to LDL-RD mice, the extent of lymph node proliferation in response to challenge with β_2GPI was compared in mice receiving oral β_2GPI or BSA administration. Figs. 7A and 7B show the differences in thymidine uptake, expressed as Stimulation Index, between

lymph node cells from LDL-RD mice immunized with β_2GPI (Fig. 7A) or oxidized LDL (oxLDL, Fig. 7B), following oral administration of β_2GPI or BSA, and exposure of the cells to the sensitizing antigen.

Oral administration of β_2GPI effectively inhibits the cellular immune response to the plaque related antigens in sensitized mice. Fig. 7a shows the significant inhibition of lymph node cell proliferation stimulated by β_2GPI in the β_2GPI tolerized mice, even at the exceedingly low doses of β_2GPI also found effective in suppressing atherosclerosis (see Fig. 7a, 2.0μg and 0.4μg/ml). Fig. 7b shows the effect of oral administration of β_2GPI on lymph node cell proliferation stimulated by oxidized LDL. The results clearly show that prior oral administration of β_2GPI (5 doses of 500 μg per mouse) suppressed the primary cellular response to stimulation with both β_2GPI (>60% suppression upon stimulation with 2.0 μg/ml β_2GPI) (Fig. 7a) and, surprisingly, also to the plaque antigen oxLDL (74% suppression upon stimulation with 20 μg/ml oxLDL)(Fig. 7b). Oral administration of β_2GPI did not influence the primary cellular response to BSA in mice immunized with BSA, and oral administration of BSA in mice immunized with oxLDL had no effect on the proliferative response to the sensitizing antigen (oxLDL) (results not shown). Thus, oral administration of β_2GPI results in specific suppression of the primary cellular immune response to β_2GPI as well as to other plaque autoantigens.

Changes in the lymph node cell cytokine profile following oral administration of β_2GPI: To investigate whether suppression of lymph node cell reactivity to β_2GPI was associated with a change in cytokine production, conditioned medium from lymph node cells collected from mice receiving oral β_2GPI or BSA administration, following immunization with β_2GPI (50 g/mouse), and incubated for 48 hours in the presence of β_2GPI, was collected, and assayed for cytokines.

IL-4 and IL-10: The levels of anti-inflammatory type Th2 cytokines IL-4 and IL-10 were measured. Levels of IL-4 in medium from cells of animals receiving oral β_2GPI were three times higher ($p < 0.01$) than those from lymph node cells from control animals (Fig. 8). A similar pattern was evident with regard to IL-10. Namely, lymph node cells from animals receiving oral β_2GPI administration following immunization with β_2GPI secreted significantly more IL-10 (2.6 times higher; $p <$

0.05) upon *in-vitro* priming with β_2GPI than did lymph node cells from BSA-treated controls (Fig. 8).

IFN-γ and TGF-β: Levels of the proinflammatory Th1-type cytokine IFN-γ and the anti-inflammatory mediator of mucosal tolerance TGF-β were measured. Oral administration of β_2GPI did not induce significant changes in the levels of IFN-γ secreted by lymph node cells in response to stimulation with β_2GPI (mean value of 1802±588 pg/ml in the cells from β_2GPI-tolerized mice as compared with 1870±378 pg/ml in cells from controls). TGF-β levels in the conditioned medium of lymph node cells obtained from β_2GPI-tolerized and non-tolerized control mice were below the detection threshold.

Oral administration of β_2GPI induces anti-inflammatory cytokines in-vivo: To determine whether the changes in cytokine profile observed in lymph node cells of β_2GPI-tolerized mice reflected significant modulation of the inflammatory response of the affected tissue *in-vivo*, the cytokine expression profile of aorta tissue from ApoE-KO mice following a regimen of mucosal administration of β_2GPI was determined by RT-PCT using primers specific to IL-10, IFN-γ, and β-actin as control.

Male ApoE-KO mice 7-9 weeks of age were treated by oral administration of human β_2GPI (100 μg/mouse) or PBS, as control, by gavage, as detailed hereinabove, 5 times, on every other day. Three days following the oral administration of β_2GPI, the mice were sacrificed, and aortas collected and processed for RT-PCR analysis of the expression of anti-inflammatory Th2 type cytokine IL-10 and the proinflammatory Th1-type cytokine IFN-γ. The results of SDS-PAGE separation of the PCR products (Fig. 9) clearly show induction of expression of the anti-inflammatory, anti-atherogenic cytokine IL-10, and inhibition of expression of the proinflammatory cytokine IFN-γ within the atheromatous regions of the aortas, without any influence on tissue levels of the housekeeping β-actin gene transcripts.

These results show, for the first time, that mucosal administration of β_2GPI to subjects genetically predisposed to atherosclerosis, suppresses immune reactivity to β_2GPI, and causes a shift in the immune profile, enhancing expression and tissue levels of anti-inflammatory cytokines and suppressing pro-inflammatory cytokines expression, in the lymph organs and in the aortic tissue itself.

Oral administration of β_2GPI does not affect antibody levels: To explore whether Th2 cytokine dominance in the lymph nodes of β_2GPI-tolerized mice was associated with a skewed antibody isotype distribution, total antibody levels as well as the anti-β_2GPI IgM, IgG and IgA antibody levels and isotypes were measured in sera of β2GPI-tolerized mice that were subsequently immunized with β_2GPI. Oral administration of β_2GPI did not alter IgM, IgA, or IgG total antibody levels nor was there a change in isotype distribution in comparison with non-tolerant mice (data not shown). None of the orally-administered antigens induced production of anti β_2GPI antibodies (data not shown).

Thus, these results indicate that oral administration of β_2GPI results in increased levels of the Th2 type cytokines IL-10 and IL-4 in response to stimulation with β_2GPI, but no difference in levels of IFN-γ or TGF-β in lymph node cells from tolerized mice. On the other hand, the effects of oral administration of β_2GPI on cytokine expression in aorta tissue *in-vivo* clearly indicate enhanced anti-inflammatory IL-10 and suppression of proinflammatory IFN-γ expression.

Taken together, the results brought hereinabove unexpectedly reveal that mucosal administration, via both oral and nasal presentation of the plaque-related antigen β_2GPI according to the methods of the present invention effectively inhibits both early and late stage atherogenic processes and, although no change in the inflammatory cell infiltration, macrophage content or antibody profile was noted, mucosal β_2GPI administration results in induction of the Th2 type cytokines and has a strong suppressive effect on reactivity of sensitized immune cells to stimulation by β_2GPI. Without wishing to be limited by a single hypothesis, it is feasible that increased levels of IL-10 results in the striking antiatherogenic effects (inhibition of the proinflammatory nuclear factor-B, inhibition of matrix metalloproteinases, reduction of tissue factor expression, and inhibition of apoptosis of macrophages and monocytes) unrelated to Th1 cytokine suppression.

Further, the results reveal, for the first time, that mucosal administration of β_2GPI prior to immunization of the mice with oxLDL significantly inhibits the primary immune responses to oxLDL stimulation. Without wishing to be limited by a single hypothesis, this tolerizing effect on oxLDL responsiveness can be mediated

through the "bystander effect", involving regulatory cells secreting nonantigen-specific cytokines that suppress inflammation in the microenvironment where the mucosally administered antigen is localized such as has been demonstrated for colon-distinctive protein (Gotesman et al, J Pharma and Expanding Ther. 2001; 297-32), pre-transplant splenocytes antigens (Ilan et al, Blood 2000; 95:3613-19) and myelin basic protein (Becker et al. PNAS USA 1997;94:10873-78).

Although the invention has been described in conjunction with specific embodiments thereof, it is evident that many alternatives, modifications and variations will be apparent to those skilled in the art. Accordingly, it is intended to embrace all such alternatives, modifications and variations that fall within the spirit and broad scope of the appended claims. All publications, patents, patent applications and sequences identified by their accession numbers mentioned in this specification are herein incorporated in their entirety by reference into the specification, to the same extent as if each individual publication, patent, patent application or sequence identified by their accession number was specifically and individually indicated to be incorporated herein by reference. In addition, citation or identification of any reference in this application shall not be construed as an admission that such reference is available as prior art to the present invention.

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WHAT IS CLAIMED IS:

1. A method for prevention and/or treatment of a vascular condition in a subject in need thereof comprising administering to a mucosal surface of the subject a mucosal tolerance-inducing amount of an active ingredient selected from the group consisting of beta$_2$-glycoprotein-1 (β_2GPI) or a derivative of β_2GPI, thereby inducing mucosal tolerance.

2. The method of claim 1, wherein said β_2GPI is human β_2GPI.

3. The method of claim 1, wherein said administering is effected by oral, enteral, buccal, nasal, bronchial, intrapulmonary or intra- peritoneal administration.

4. The method of claim 1, further comprising administering a therapeutic amount of at least one additional compound selected from the group consisting of HMGCoA reductase inhibitors (statins), mucosal adjuvants, corticosteroids, anti-inflammatory compounds, analgesics, growth factors, toxins, and additional tolerizing antigens.

5. The method of claim 1, wherein said vascular condition is selected from the group consisting of atherosclerosis, cardiovascular disease, cerebrovascular disease, peripheral vascular disease, stenosis, restenosis and/or in-stent-stenosis.

6. A method for modulating an immune response to a beta$_2$-glycoprotein-1 (β_2GPI) in a subject in need thereof comprising administering to a mucosal surface of the subject a mucosal tolerance-inducing amount of an active ingredient selected from the group consisting of beta$_2$-glycoprotein-1 (β_2GPI) or a derivative of β_2GPI, thereby inducing mucosal tolerance and modulating the immune response to the β_2GPI.

7. The method of claim 6, wherein said β_2GPI is human β_2GPI.

8. The method of claim 6, wherein said modulating is reducing immune reactivity to β_2GPI in the subject.

9. The method of claim 6, wherein said immune response is selected from the group consisting of Th1 type cytokines expression, Th2 type cytokines expression, and T-cell proliferation.

10. The method of claim 6, wherein said administering is effected by oral, enteral, buccal, nasal, bronchial, intrapulmonary or intra- peritoneal administration.

11. The method of claim 6, further comprising administering a therapeutic amount of at least one additional compound selected from the group consisting of HMGCoA reductase inhibitors (statins), mucosal adjuvants, corticosteroids, anti-inflammatory compounds, analgesics, growth factors, toxins, and additional tolerizing antigens.

12. A method for modulating an immune response to an atheroma plaque-related antigen in a subject in need thereof comprising administering to a mucosal surface of the subject a mucosal tolerance-inducing amount of an active ingredient selected from the group consisting of beta$_2$-glycoprotein-1 (β_2GPI) or a derivative of β_2GPI, thereby inducing mucosal tolerance and modulating the immune response to the atherosclerotic plaque antigen.

13. The method of claim 12, wherein said β_2GPI is human β_2GPI.

14. The method of claim 12, wherein said atheroma plaque-related antigen is selected from the group consisting of beta$_2$-glycoprotein-1 (β_2GPI), oxidized LDL (oxLDL) and heat shock protein (HSP 60/65).

15. The method of claim 12, wherein said modulating is reducing immune reactivity to the atherosclerotic plaque-related antigen in the subject.

16. The method of claim 12, wherein said immune response is selected from the group consisting of Th1 type cytokines expression, Th2 type cytokines expression, and T-cell proliferation.

17. The method of claim 12, wherein said administering is effected by oral, enteral, buccal, nasal, bronchial, intrapulmonary or intra- peritoneal administration.

18. The method of claim 12, further comprising administering a therapeutic amount of at least one additional compound selected from the group consisting of HMGCoA reductase inhibitors (statins), mucosal adjuvants, corticosteroids, anti-inflammatory compounds, analgesics, growth factors, toxins, and additional tolerizing antigens.

19. An article of manufacture, packaged and identified for use in the prevention and/or treatment of a vascular condition in a subject in need thereof comprising a packaging material and a mucosal tolerance-inducing amount of an active ingredient selected from the group consisting of beta$_2$-glycoprotein-1 (β_2GPI) or a derivative of β_2GPI, and wherein said packaging material comprises a label or package insert indicating that said mucosal tolerance-inducing amount of said active ingredient is for prevention and/or treatment of a vascular condition in the subject via mucosal administration.

20. The article of manufacture of claim 19, wherein said β_2GPI is human β_2GPI.

21. The article of manufacture of claim 19, wherein said vascular condition is selected from the group consisting of atherosclerosis, cardiovascular disease, cerebrovascular disease, peripheral vascular disease, stenosis, restenosis and/or in-stent-stenosis.

22. The article of manufacture of claim 19, further comprising a therapeutically effective amount of at least one additional compound selected from the group consisting of HMGCoA reductase inhibitors (statins), mucosal adjuvants, corticosteroids, anti-inflammatory compounds, analgesics, growth factors, toxins, and additional tolerizing antigens.

23. An article of manufacture, packaged and identified for use in modulating an immune response to an atheroma plaque-related antigen in a subject in need thereof comprising a packaging material and a mucosal tolerance-inducing amount of an active ingredient selected from the group consisting of beta$_2$-glycoprotein-1 (β_2GPI) or a derivative of β_2GPI, and wherein said packaging material comprises a label or package insert indicating that said mucosal tolerance-inducing amount of said active ingredient is for modulating an immune response to an atherosclerostic plaque antigen in the subject via mucosal administration.

24. The method of claim 23, wherein said atheroma plaque-related antigen is selected from the group consisting of beta$_2$-glycoprotein-1 (β_2GPI), oxidized LDL (oxLDL) and heat shock protein (HSP 60/65).

25. The article of manufacture of claim 24, wherein said β_2GPI is human β_2GPI.

26. The article of manufacture of claim 23, wherein said vascular condition is selected from the group consisting of atherosclerosis, cardiovascular disease, cerebrovascular disease, peripheral vascular disease, stenosis, restenosis and/or in-stent-stenosis.

27. The article of manufacture of claim 23, further comprising a therapeutically effective amount of at least one additional compound selected from the group consisting of HMGCoA reductase inhibitors (statins), mucosal adjuvants, corticosteroids, anti-inflammatory compounds, analgesics, growth factors, toxins, and additional tolerizing antigens.

28. The article of manufacture of claim 23, wherein said immune response is selected from the group consisting of Th1 type cytokines expression, Th2 type cytokines expression, and T-cell proliferation.

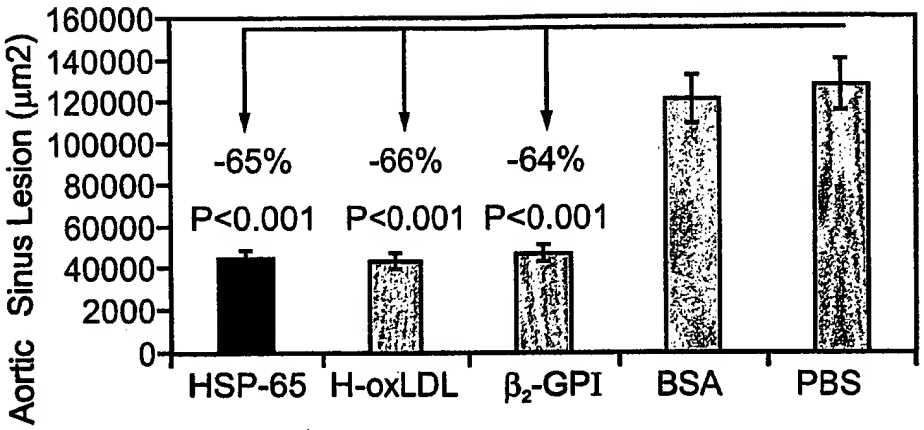

Aortic Sinus Lesion (µm2)
160000
140000
120000
100000
80000
60000
40000
2000
0
-65%
-66%
-64%
P<0.001
P<0.001
P<0.001
HSP-65
H-oxLDL
β2-GPI
BSA
PBS

Fig. 1

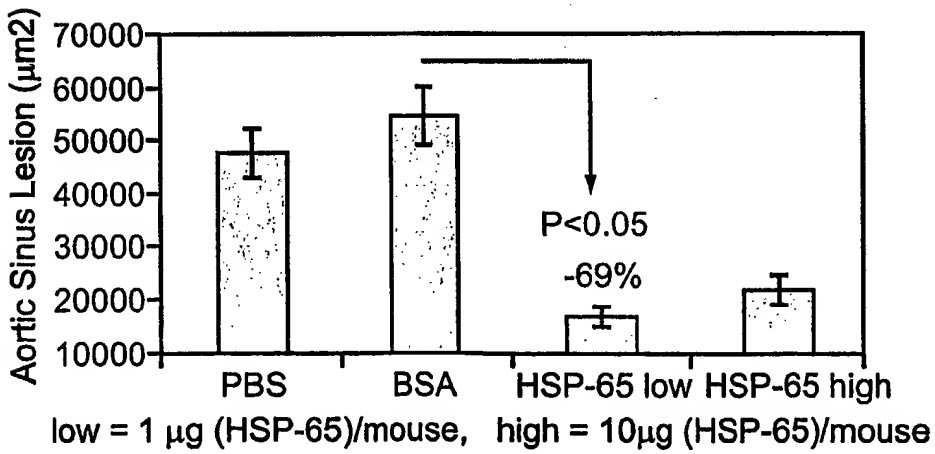
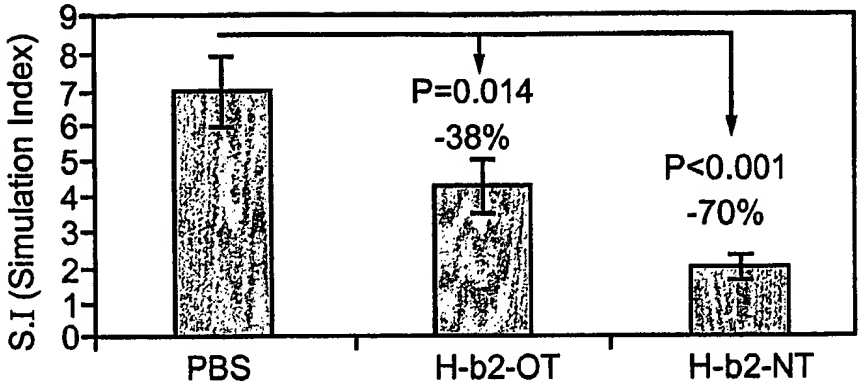

Aortic Sinus Lesion (µm2)
70000
60000
50000
40000
30000
20000
10000
P<0.05
-69%
PBS
BSA
HSP-65 low
HSP-65 high
low = 1 µg (HSP-65)/mouse, high = 10µg (HSP-65)/mouse

Fig. 2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8
7
6
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4
3
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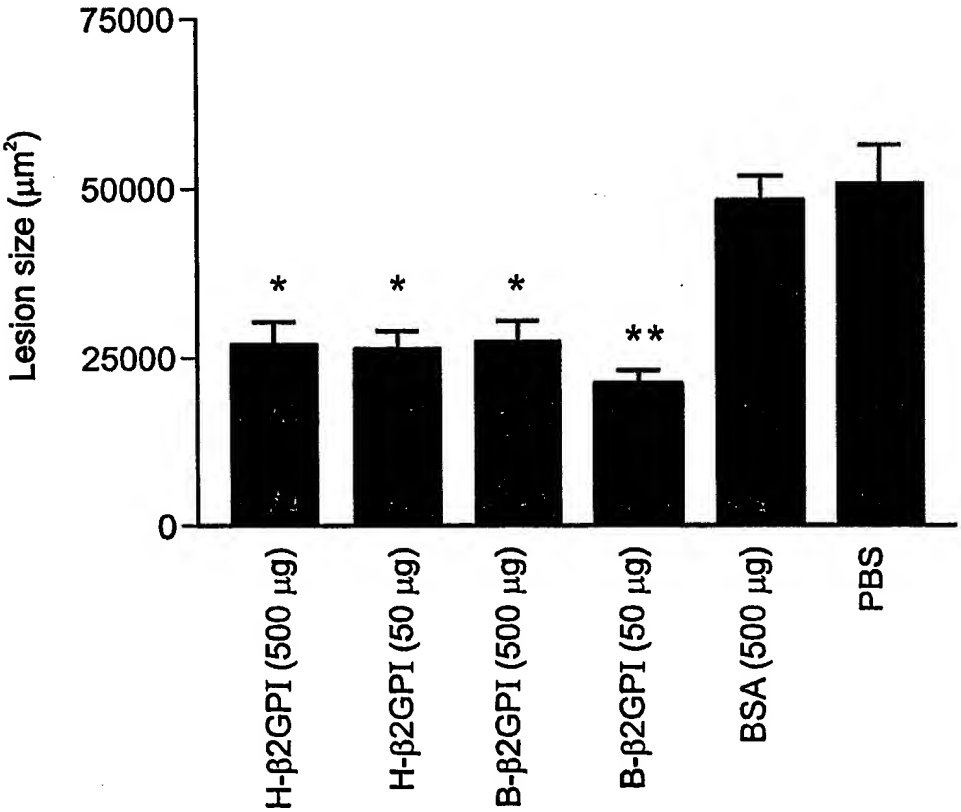
P<0.001
-70%
PBS
H-b2-OT
H-b2-NT

Fig. 3


Lesion size (μm²)
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50000
25000
0
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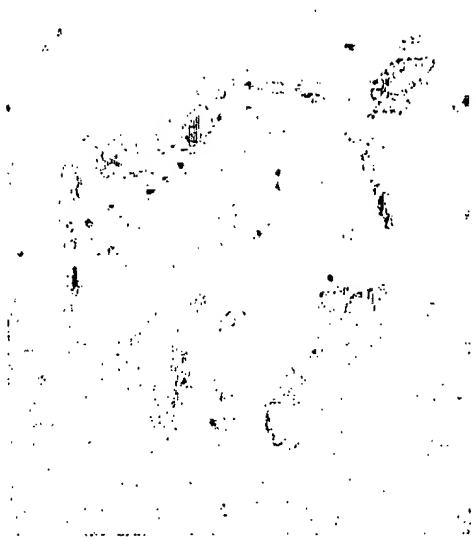
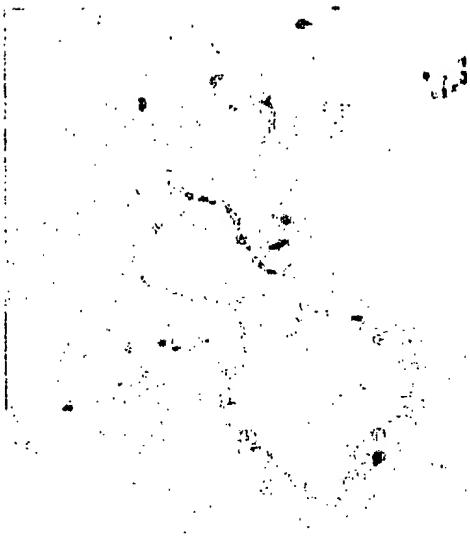
*
*
**
H-β2GPI (500 μg)
H-β2GPI (50 μg)
B-β2GPI (500 μg)
B-β2GPI (50 μg)
BSA (500 μg)
PBS

Fig. 4



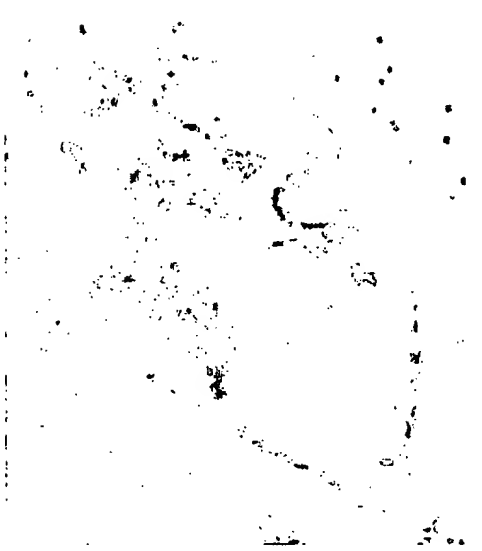
PBS

Fig. 5a



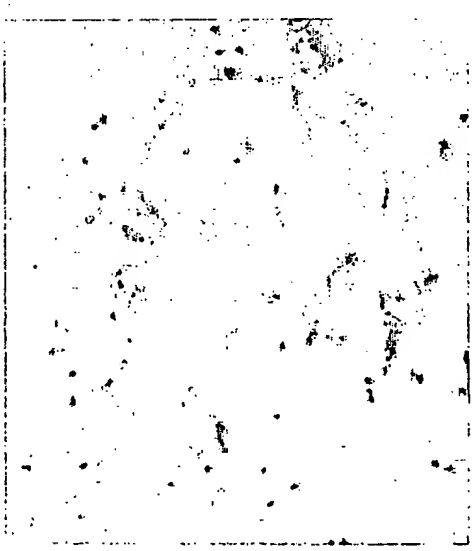
BSA

Fig. 5b



Bovine-β_2 GPI-1 50 µg/mouse

Fig. 5c



Human-β_2 GPI-1 50 µg/mouse

Fig. 5d

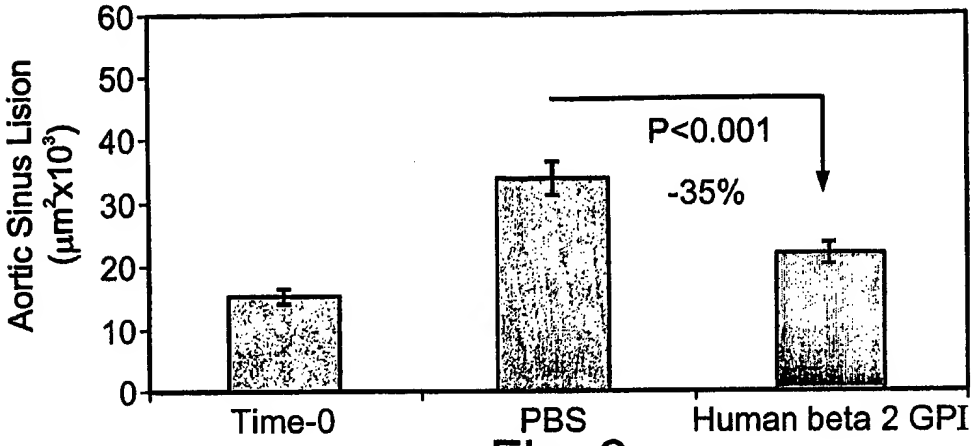


Fig. 6

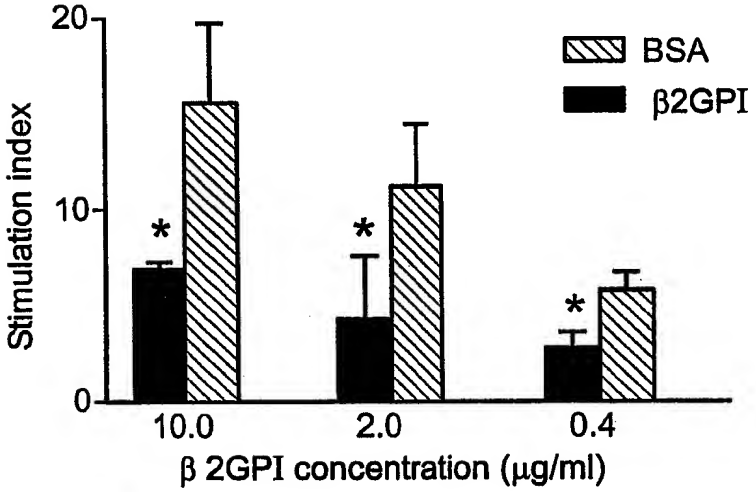


Fig. 7a

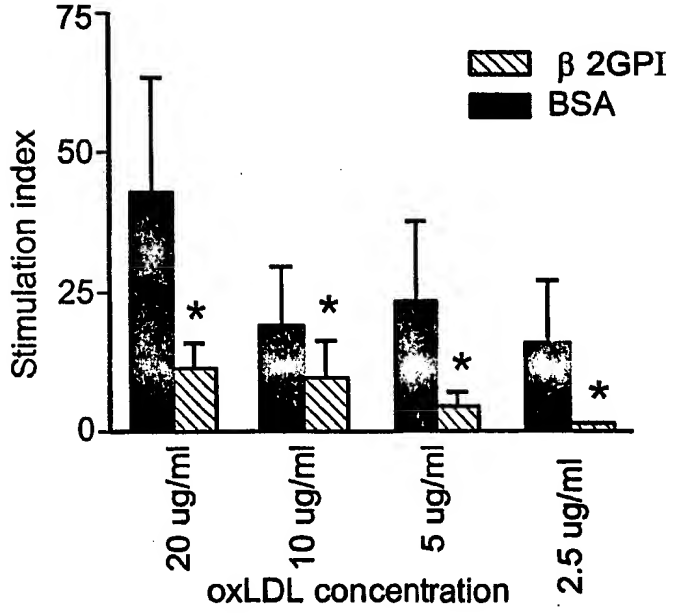
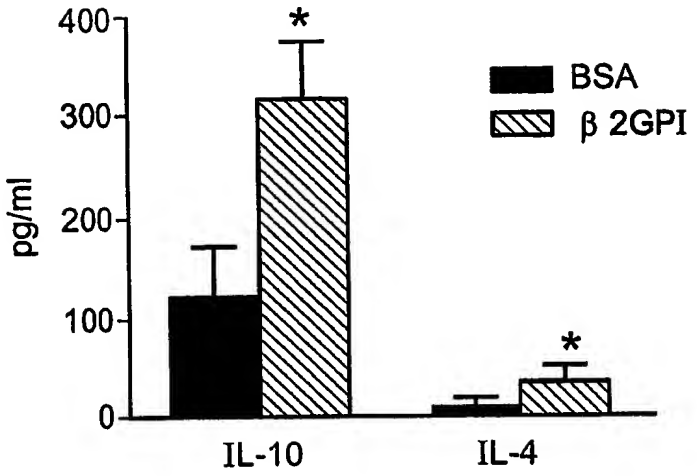


Fig. 7b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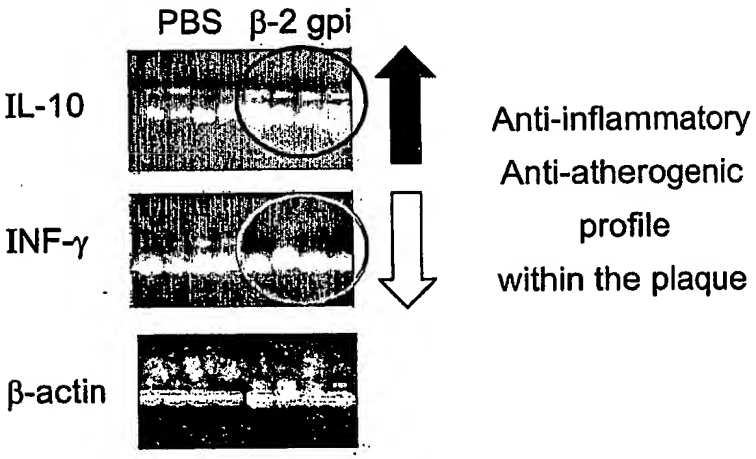
300
200
100
0
pg/ml
*
*
BSA
β 2GPI
IL-10
IL-4

Fig. 8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IL-10
INF-γ
β-actin
Anti-inflammatory
Anti-atherogenic
profile
within the plaque

Fig. 9

SEQUENCE LISTING

<110> Harats, Dror
Shoenfeld, Yehuda
Yacov , Niva
George, Jacob

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/IL05/01022

A. CLASSIFICATION OF SUBJECT MATTER
IPC: C07K 14/00(2006.01),14/745(2006.01);A61K 38/16(2006.01),38/36(2006.01)

USPC: 514/2;530/350
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
U.S. : 514/2; 530/350

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
WEST 2.1, MEDICINE/BIOTECH (compendium databases on DIALOG)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 02/053092 A2 (CARDIMMUNE LTD.) 11 July 2002, see entire document.	1-28

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:

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"E" earlier application or patent published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

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"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
20 February 2006 (20.02.2006)	28 MAR 2006
Name and mailing address of the ISA/US Mail Stop PCT, Attn: ISA/US Commissioner for Patents P.O. Box 1450 Alexandria, Virginia 22313-1450 Facsimile No. (571) 273-3201	Authorized officer Ron Schwadron, Ph.D. Telephone No. 571 272 1600

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